

nature

October 28, 1976

Dear Mrs Williams . . . HELP

BRITAIN'S science funding through the research councils has got itself into a real mess these past few weeks. Shirley Williams, Secretary of State for Education and Science, whose stock is already high amongst both administrators and scientists, could well earn herself even more credit by managing to sort out a log-jam which no one else has the political clout to do.

Faced like every other body with the need for economic restraint, the Advisory Board for the Research Councils (ABRC) has been saying for some time now, and most notably in its Second Report published about six months ago, that if expenditure is allowed to grow at all it is the research councils other than the Science Research Council (SRC) which will get any growth that is going. Indeed, to help fund this the SRC has had to expect to spend 2% less per year over the years to 1980. This makes the period 1973-80 one in which the spending power of the SRC in real terms is expected to fall by one-sixth.

Within that overall decline, moreover, there were to be favoured areas. Engineering research and associated schemes such as the Teaching Company, marine technology and polymer engineering, were to grow by more than 50% in the years 1976-81. Science Board research comprising biology, chemistry, mathematics and physics outside astronomy and nuclear physics is allowed a 10% growth in the same period, although all of this and more will be taken up by expansion of central facilities. On the other hand nuclear physics expenditure (at present £41 million per annum, half domestic, half at CERN) will drop to £29.5 million, with all domestic expenditure nearly halved. And expenditure in astronomy and space research will drop from £26 million to £20 million, again with the subscription to the European Space Agency assuming an increased importance. Whatever abuse may be hurled at SRC, it has certainly shown itself capable of rapid response to outside pressures for changes in direction—some of these switches mean that laboratories are having to change their rôle entirely.

Now all of this makes for a most remarkable contrast in reading between the SRC's annual report, just published (HMSO: £1.75) and the reports of the Natural Environment Research Council — NERC (HMSO: £2.65) — and the Medical Research Council — MRC (HMSO: £2.75). (Even the price difference tells the

story). Whereas NERC and MRC, although generally worried by economic stringency, seem to find their biggest problems in such matters as adapting to the customer-contractor relationship and giving their researchers a stable career, SRC looks burdened down on almost all sides. But it is what has happened since the end of the reporting year (31st March, 1976) which now has everyone so much on edge. Changes in the value of the pound have put up the cost of the international subscriptions to just those agencies such as CERN and ESA that are meant to take the load off the domestic budget. Of the £6 million more that SRC needs to find, the Treasury has as yet only agreed to cover £2.5 million.

The SRC has a long way to go, and for the past three weeks has been seeing what can be trimmed, in a fashion which has put even more people on edge. Requests have gone out to directors of establishments to look at their capital expenditures with a view to making immediate savings. Major establishments will have to cut capital expenditure by a fifth in the next year and one round of new research grants, due in a month's time, is going to be deferred. The other research councils are also having to bear the load of CERN and ESA.

No one would pretend that all this unseemly grubbing around is going to bring Britain's science to a grinding halt within months. But it does seem to have generated a fair amount of unnecessary bad feeling amongst scientists. And it does pose problems about whether the whole structure of scientific research—big science and little science alike—should be so easily rocked by international exchange rates.

In the short term, it is incumbent on Mrs Williams, convinced European that she is, to go in fighting for automatic reimbursement of the increased European subscriptions. But in the longer run there is no doubt that some way must be found of giving the chairmen of research councils increased flexibility in their transactions. As it is they are committed every year down to their last penny and have no source of stand-by credit on which they could draw in situations such as these and which they could repay gradually if special pleading found no favour with the Treasury. □

● In *Nature* of September 30, page 359, the maximum permissible body burden for plutonium was given as 0.6 mg. This should, of course, have been 0.6 μ g.



The Nobel prizes (1): Physics

Pioneers of the New Physics

Stuart Sharrock puts the achievement of Richter and Ting into perspective

THE discovery of stable elementary particles of high mass has opened up a new era in experimental and theoretical particle physics. For their pioneering work in this discovery Professor Burton Richter of the Stanford Linear Accelerator Center (SLAC) and Professor Samuel Ting of the Massachusetts Institute of Technology have been jointly awarded the 1976 Nobel Prize for physics.

The existence of a new particle was announced simultaneously by the two experimental teams in November 1974. It was observed independently by Ting's group working on the proton accelerator at the Brookhaven National Laboratory (BNL), and by Richter's team at the electron-positron storage ring facility at SLAC. The new particle, called J at BNL and ψ at SLAC, is a neutral meson of high mass, 3.1 GeV, and with the amazingly narrow width of 100 keV. It is difficult to detect with proton machines, and its discovery at BNL was a great feat of experimentation. In contrast it is copiously produced in electron-positron annihilation, although its narrow width makes it elusive. Within a few days of the announcement of the J/ψ the SLAC team had discovered a further state, the ψ' at 3.7 GeV.

The existence of stable particles of high mass was unexpected. The J/ψ is a spin 1 particle and is more than twenty times heavier than the lightest such particle, the pion. The J/ψ should rapidly decay into lighter known particles, yet its lifetime (related to its width through the uncertainty principle) is about 1,000 times longer than expected. Furthermore the J/ψ does not fit into the well-established three-quark classification scheme. A new law of nature is therefore necessary to explain its decay properties and extremely long lifetime.

Of the four known forces between particles only the electromagnetic interaction is understood. Both Richter and Ting have in the past contributed to this understanding by demonstrating the validity of the theory down to distances of the order of 10^{-14} cm. Attempts at a unified field theory for the weak and electromagnetic interactions based on the principle of 'gauge invariance', in which an additional symmetry is assumed to exist between the two interactions, have had some success in recent years, particularly with the prediction and subsequent

observation of the neutral current weak interactions. The theories require the existence of a fourth quark, distinguished by the name 'charm'. This new quantum number has successfully explained the J/ψ particle as a bound state of a charmed quark and a charmed anti-quark.

If the charmed quark is heavy then the long lifetime of the J/ψ is explained. The bound state of charm-anticharm is similar to that of the e^+e^- system in positronium, and is called charmonium. A number of energy levels are expected in this model and many states have now been found fitting well into the predicted level scheme. The investigation of charmonium promises to play a key role in the understanding of strong interactions by providing information on the force acting between quarks.

Particles are classified as hadrons if they have strong interactions and leptons if they do not. The addition of a charmed quark to the three conventional quarks provides a set of building blocks from which all known hadrons can be constructed. It also provides a symmetry between leptons and quarks, and there are now four of each with interactions that can be expressed in terms of the same set of mathematical rules. The detection of the new particles has given rise to a new branch of hadron physics which could lead to a better understanding of the symmetries governing the structure of elementary particles. Just as the discovery of neutral currents may help to unify the weak and electromagnetic interactions, the concept of a charmed quark could well indicate links between the weak and strong interactions.

Professor Ting's experiment at BNL was part of a systematic and precise study of lepton pairs produced in hadron-hadron collisions. This study was started in 1972 and, to cover the mass region 1-50 GeV, involved experiments at BNL, the ISR in Geneva and the electron synchrotron DESY in Hamburg. One of the aims was to search for long-lived particles decaying into lepton pairs. These experiments are very difficult as the rate of electromagnetic pair production in hadron-hadron collisions is extremely low compared to the rate of production of hadron pairs. The experiment had to reject hadron pairs by a factor of at least 100 million. During the period August-October 1974 the Ting group

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AP

Samuel Ting

had seen a narrow peak in the effective mass distribution of electron pairs, and had performed many detailed checks to prove that the peak was a real particle. The announcement of the discovery was withheld at first while the experiment was modified to search for possible further new phenomena. The inevitable rumours began to circulate and the group decided to publish at the beginning of November. Around this time the new particle was also seen at SLAC and the results of the two experiments were published simultaneously.

At SLAC, Richter's team had been working with the immense magnetic detector at the electron-positron storage ring complex (SPEAR). They had compiled a large volume of data on the total cross-section for e^+e^- annihilation as a function of energy. On checking through these data an anomaly was observed at one particular energy setting: inconsistent values of the total cross-section were obtained in measurements taken at the same nominal energy. The group decided this anomaly had to be cleared up, and at the beginning of November they decided to investigate the energy region in detail. Within hours of starting an extremely narrow peak in the

cross-section had been seen. This effect was so strong and clear that the group immediately sat down and wrote the draft of their paper announcing the discovery of the new particle. The following day Richter and Ting met at a committee meeting and to their mutual astonishment started to tell each other about the interesting physics results they had found.

Burton Richter was born in 1931 in New York. He decided to become a scientist as a child in high school, a physicist as an undergraduate, and a particle physicist after obtaining his PhD from MIT in 1956. Richter, always fascinated by electrons, sought a job at SLAC where he performed experiments demonstrating the range of validity of quantum electrodynamics

down to very small distances. He has long been a proponent of the virtues of electron-electron scattering and was instrumental in the commissioning of the e^+e^- colliding beam machine at SLAC. During this time he designed an e^+e^- storage ring and worked for ten years to create the complex known as SPEAR. His team working on SPEAR began experiments in 1973 culminating in the discovery of the ψ family of particles and the subsequent measurement of their properties.

Samuel Ting was born in 1936 in Michigan but returned to China with his parents when he was two months old. At the age of 20 he returned to America with \$100, very little knowledge of English and a determination to go to university. Within a short time

he had obtained a degree and a PhD from the University of Michigan and decided to become an experimental physicist. After a year at CERN he returned to teach at Columbia University in 1965. At Columbia he proposed an experiment at DESY to check a recent experimental result which appeared to show a violation of quantum electrodynamics. This was accepted, and in 1966 he began a long association with DESY in which he performed experiments extending the range of validity of quantum electrodynamics and determining the properties of vector mesons. His work over the last decade on lepton production culminated in the discovery of the J/ψ by its decay into electron pairs. □

The Nobel prizes (2): Chemistry

Boranes surgery

A Special Correspondent looks at the work of William Lipscomb

IT is no surprise to chemists that Bill Lipscomb should have been awarded the Nobel Prize for his beautiful work on the boron hydrides. The layman may, however, be baffled, wondering what these oddly-named entities might be, and why they should have merited years of study by a leading crystallographer who is no less well known for his more recent work on the structure of enzymes.

The story goes back more than half a century, to the time when the great inorganic chemist Alfred Stock reported that the simplest compound of boron and hydrogen is not BH_3 , as one would expect by analogy with BF_3 , but its dimer B_2H_6 . The trouble with B_2H_6 , and with all the other boron hydrides which he described, was its "electron deficiency". If the molecule had an ethane-like geometry (as the evidence of electron diffraction was held to indicate) it would need 7 electron-pair bonds to hold its atoms together, but only 12 valency electrons were available for the purpose. This ominous crack in orthodox valency theory was papered over by Linus Pauling, who advanced special reasons why the B-H bond should be able to make do with less than 2 electrons, and proposed structures for the higher boron hydrides in line with this idea.

In 1943 Longuet-Higgins and Bell showed that the chemical and spectroscopic evidence on B_2H_6 harmonised less well with an ethane-like structure than with a bridge structure, in which

two of the H atoms are situated in the middle of the molecule. It appeared that B_2H_6 manages to hold together, not by starving all its bonds of electrons but by sharing two electron pairs between the two sides of the central bridge. The bridge structure was confirmed soon afterwards by W. C. Price, and in 1949 it was proposed that one should regard B_2H_6 as containing two "liaisons triatomiques", or "3-centre bonds", neatly describable in terms of localised molecular orbitals. But the higher boron hydrides remained a puzzle.

At this point the crystallographers grasped the initiative, and Kasper, Lucht and Harker led off with a definitive determination of the molecular structure of crystalline $B_{10}H_{12}$. Their results if anything deepened the mystery: the bonding in this molecule clearly involved principles more general than those which had been advanced for B_2H_6 , and it became clear that a full understanding of these principles must await the collection of structural evidence on the other boron hydrides.

It was Lipscomb who took up this challenge. Undaunted by the pathological instability of most boron hydrides and the acute difficulty of "seeing" hydrogen atoms by X-ray diffraction, he and his colleagues completed a sequence of brilliant experimental studies of the hydrides described by Stock, and of others isolated in his own laboratory. A pattern soon

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William Lipscomb

began to emerge which could be interpreted in terms of "3-centre bonds" of various novel kinds, together with "many-centre bonds", for whose possible existence there were solid grounds in molecular orbital theory. Simultaneous theoretical studies in England and by Lipscomb's group predicted the existence of a stable ion $B_{12}H_{12}^{--}$ with icosahedral symmetry.

The preparation of the potassium salt, and the subsequent determination of its structure by Wunderlich and Lipscomb in 1960, set the final seal on the molecular orbital theory of the bonding in these remarkable substances. The fascinating work of Lipscomb and his colleagues is a story of the fruitful interplay between imaginative theory and skilled experiment, leading to a genuine "breakthrough" in our understanding of the forces which bind atoms together. No longer is there a dichotomy between the chemist's "covalent" bond and the "metallic" bond of the physicist: Lipscomb's work showed us that at least one element succeeds, in its hydrides, in forming both kinds of bond within the same molecular species. □

The Nobel prizes (3): Medicine

Antipodean . . .

Carleton Gajdusek's attainments stretch beyond virology. **Cedric Mims** outlines his work and assesses its significance.

CARLETON Gajdusek came into medical virology in the early 1950s and he soon developed a particular interest in the haemorrhagic fevers of the USSR and the Far East. His work on virus infections took him to many other parts of the world including South America, the Middle East and a number of Pacific Islands. Not content merely to investigate the viruses infecting a given population he also made extensive studies of the behaviour, development and language of the people. In all but name he became an anthropologist. During the 1960s he could be counted on to talk with authority not only about the exotic diseases occurring in peoples in remote corners of the earth, but also about aspects of their physical and cultural anthropology. In 1959, at the National Institute of Health in Bethesda, Maryland, he became simultaneously director of a programme for studying child growth and development in primitive cultures and director of the laboratories for slow, latent and temperate virus infections.

It was in 1957, during an expedition to the highlands of New Guinea, that he encountered the disease kuru, and together with Vincent Zigas, the local physician who already knew the disease, he brought kuru to the attention of the rest of the world. Kuru was a chronic neurological condition, progressive and fatal, affecting especially the cerebellum and occurring in a sharply localised area inhabited by the Fore tribe. In these people it was responsible for more than half the total deaths after infancy, and in some villages at least half the women were affected.

Initial thoughts about the role of local genes, local plant or other toxins, were dramatically swept away after Dr Hadlow, of the Rocky Mountain Laboratory, Montana, had pointed out that the brains of patients with kuru bore a histological resemblance to the brains of animals suffering from scrapie. Scrapie, another chronic degenerative neurological condition affecting sheep, was known to be transmissible experimentally from animal to animal, but only after an unusually long incubation period of a year or two. Gajdusek therefore flew refrigerated brains from patients with kuru to the Washington laboratory where in August 1963 his close col-

laborator, Joe Gibbs, injected the brain suspensions into two chimpanzees. Twenty-one and thirty months later the injected animals were seen to sicken with a disease closely similar to kuru and their brains showed almost identical pathological changes.

The disease could be transmitted from chimpanzee to chimpanzee, in whom the average incubation period was 22 months, and in subsequent experiments various other primates were found to be susceptible. All the usual laboratory tests for microorganisms were negative, except for a number of chimpanzee viruses which were recovered and appeared to represent the normal viral flora of the brain. The transmissibility of the agent from animal to animal and its assay ($10^{7.5}$ infectious doses present per gram of brain) firmly established that kuru was caused by an infectious agent. But kuru, being closely similar in its properties to scrapie, was not a conventional virus and to this day its composition and mechanism of replication is unknown. For the first time therefore, a chronic neurological condition of man, in which there were no pathological or other signs of infection, had been shown to be due to a transmissible agent.

‘It seems probable that we have still not gathered the full harvest of the discoveries made by Gajdusek’

Throughout this work and during his frequent visits to New Guinea, Gajdusek had both the opportunity and the energy to exercise his anthropological interests. As a result of this he wrote various monographs, produced films and contributed to paediatric journals. The marriage of virology and anthropology bore appropriate fruit when it became clear that the disease kuru in New Guinea was transmitted from person to person by cannibalism. The disappearance of cannibalism in this part of New Guinea has meant that kuru too has now almost disappeared.

Kuru is restricted to a small zone

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Carleton Gajdusek

in the highlands of New Guinea, where a total of some 2,000 cases has been reported since its discovery in 1957. But the discoveries about kuru have sparked off a fresh approach to other chronic degenerative neurological conditions of man which are of unknown aetiology, such as amyotrophic lateral sclerosis and parkinsonian dementia. So far one of these conditions, called Creutzfeldt Jacob disease, has been shown in Gajdusek's laboratory to be transmissible to primates and caused by a replicating infectious agent similar to kuru. Creutzfeldt Jacob disease is rare, but the infectious agent has now been recovered from the brains of patients in five continents of the world. It seems quite likely that the neurological disease is the tip of the iceberg and reflects widespread sub-clinical infection.

At about the same time as the discovery of kuru, certain conventional viruses had been shown by others to be responsible for obscure and chronic neurological conditions. Measles virus was recognised as a cause of the rare disease subacute sclerosing panencephalitis, and JC virus, a new human papovavirus, came to be associated with progressive multifocal leucoencephalopathy. Once again the infectious agent was seen to persist in the body and cause a disease after a period of several years.

It is against this background that Gajdusek's work on kuru must be assessed. It is now firmly established that chronic diseases of man, especially neurological diseases, can arise as a result of the slow but relentless multiplication of certain infectious agents, sometimes referred to as "slow viruses", which cause disease many years after initial infection. The possible incubation period with kuru, for instance, is between 10 and 15 years. Much of the recent excitement in multiple sclerosis research arises from observations based

on this concept. It represents a great step forward in our understanding of disease and it seems probable that we have still not gathered the full harvest of the discoveries made by Gajdusek. These discoveries, moreover, may well prove to be of more than mere biological interest, because traditionally microbiology has moved from the recognition of an infectious aetiology to the development of a vaccine and thus to the prevention of the disease.

The agents responsible for kuru, Creutzfeld Jacob disease and scrapie pose major problems for the investigator. All assays and tests depend on the lengthy incubation period in experimental animals and it may take more than a year to learn the result of a single experiment. Other microbiologists would have shied away but Gajdusek's insight, patience and persistence have ensured that the infectious origin of kuru was discovered and the

disease given an important place in our understanding. He has been the pioneer and the central authority in this area of biomedical research. His encyclopaedic memory, his familiarity with strange places and primitive peoples have generated many legends. He is the sort of man one might have expected to win a Nobel prize and at the same time it is both a surprise and a pleasure to find such a gentle and likeable man behind the legends. □

... encounters

A **Special Correspondent** describes the discovery made by Baruch Blumberg, 'one of the success stories of the century'

SURPRISE, suspense and success are the three indispensable ingredients in a good detective story, and none of them is absent from the events which led up to the discovery and evaluation of Australia antigen, now usually known as hepatitis B antigen, or HB antigen.

The story starts nearly 20 years ago, when Dr Baruch Blumberg, one of the recipients of the 1976 Nobel Prize for Medicine, was at work on proteins in the blood. It is well known that humans show individual variation in the antigens, or blood group substances, on their red blood cells. Blumberg set out to detect and compare comparable substances in the plasma as opposed to those on the cells. Using as antisera specimens from patients who had received many transfusions, a reacting antigen was found in an Australian aborigine.

The surprises began when this was found to be widely, if unevenly, distributed in human blood throughout the world. The United States, where Dr Blumberg was working, was in fact relatively free from it, although a survey of patients revealed that cases of leukaemia, Down's syndrome (mongolism) and hepatitis figured among those who were positive. The question began to be asked: was this in fact not a simple protein antigen but a human leukaemia virus?

The possibility of being able to detect and study such a virus opened wide horizons—but it was now that the suspense began, because it quickly became unlikely that it was, in fact, a leukaemia virus. A common factor in leukaemia and Down's syndrome is altered cellular immunity; in addition, patients with leukaemia have many transfusions with a concomitant oppor-

tunity for acquiring hepatitis, and children with Down's syndrome (at least, those of them in institutions) are notoriously prone to hepatitis.

It was about this time that hepatitis B infection, acquired from transfused blood, broke upon the haemodialysis trade. The transmission of hepatitis B virus, undetectable by any means except the production of the disease in a patient, had always been a problem in blood transfusion and the use of blood products, but the disasters which overtook staff and patients in dialysis units had made it doubly urgent to find a means of recognition, a serological marker for the presence of the agent.

It is now well known how, during the late 1960s, it became clear that Australia antigen was such a marker—but was it, in fact, the hepatitis B virus? When examined by negative staining it did, so to speak, come to life, and at least some of the particles could be seen to be acceptable as virus. By now it has been possible to purify these particles, and to determine that they have double-stranded DNA and a DNA polymerase, and in fact to do many things with them except grow them in a laboratory system.

Many medical microbiologists must have hoped that this would give a clue to growing hepatitis B virus in a cell culture system, but so far they have been disappointed. However, this has not prevented the use of HB antigen to study the natural history of hepatitis B virus, to detect carriers, and, to detect antibodies in human serum, and this is where the third ingredient, a successful conclusion, comes in.

As a corollary, it is possible to prevent many cases of hepatitis B (post-transfusion and otherwise) and hence to make haemodialysis, blood transfusion,

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Baruch Blumberg

and the use of blood products infinitely safer. Unfortunately, there is still no conventional small laboratory animal, but the chimpanzee serves as a possible, if expensive, source and host. Even a vaccine has been prepared from HB-antigen-positive human blood, and even though its worth has yet to be assessed, there is little doubt about the effectiveness of the passive immunity conferred by the injection of concentrated, purified, specific anti-HB immunoglobulin.

Although the final evaluation of HB antigen involved, in the end, many laboratories, the work all springs from the researches of one principal and primary investigator. Even if, by hindsight, the connection between the molecular genetics of polymorphism and the prevention of hepatitis B in haemodialysis units all over the world seems to involve a series of knight's moves in chess, the consequences of Blumberg's discovery, judged as a piece of preventive medicine, must rank as one of the success stories of the century. Perhaps in this respect it is as near as any previous award to Alfred Nobel's desire to reward those whose discoveries have been of benefit to mankind. □

USA

Now New York steps in

The New York State Attorney General's office is considering whether or not to impose controls on recombinant DNA experiments performed in facilities in the State. Colin Norman reports from New York

A windowless hearing room on the 47th floor of New York City's World Trade Center last week provided a somewhat unlikely venue for another round of public hearings on the potential risks and benefits associated with recent advances in genetic engineering. The hearing marks a potentially important development in the long process of regulating recombinant DNA research in the United States, for it is the first time that the matter has been taken up by a state government.

Called by the New York State Attorney General, Louis J. Lefkowitz, the hearing was designed to elicit the views of various concerned individuals—mostly scientists who have been in the thick of the debate over the potential hazards of recombinant DNA research for the past two years—on whether New York should impose its own controls on the research and, if so, just how strict those controls should be.

The crux of the discussion was concerned with two closely related issues. First, whether the guidelines, published by the National Institutes of Health last June to control recombinant DNA experiments which NIH supports, are strict enough to guard against possible public health hazards. And second, whether or not the guidelines should be enforced in New York State by law.

The outcome of the discussions in New York will be closely watched elsewhere in the United States, for other states are likely to take up the matter at some stage. Already, the City Council of Cambridge, Massachusetts, has imposed a temporary moratorium on a few types of recombinant DNA experiments at Harvard and Massachusetts Institute of Technology, while a special review board, consisting of city residents, is looking into the risks and benefits associated with the research. (The Cambridge moratorium was due to expire on October 7, but it has quietly been extended until January 7 to allow the board more time to make its recommendations.) And a committee of the City Council in San Diego has also

been taking evidence from both supporters and opponents of the research. New York is the first state to consider adopting legal controls, however.

According to one official in the Attorney General's office, a major factor which led New York to look into the matter is the fact that the NIH guidelines apply only to NIH grantees, and there are no legal sanctions which can be applied if the guidelines are violated. Research supported by industry, and even by other government agencies, is not formally covered. Thus a prime consideration in New York is whether or not the controls should be backed by the force of law and made to apply to all facilities, including industrial laboratories.

Last week's hearings consisted of a parade of about a score of witnesses who urged the State government to undertake a variety of measures ranging from doing nothing to banning virtually all recombinant DNA experiments in the state. The hearings were mostly low-key, punctuated by some fairly gentle questioning by officials in the Attorney General's office. They contrasted rather starkly with the highly charged, emotional nature of the Cambridge City Council's hearings earlier this year.

Leading the call for a moratorium on the research were George Wald of Harvard, Leibe Cavalieri of the Sloan-Kettering Institute, Erwin Chargaff of Columbia University and Jonathan King of Massachusetts Institute of Technology. Cavalieri kicked off with a critique of the guidelines and the way in which they had been developed, arguing that there had been only "token" input from the public, and suggesting that "it is up to society to see that the research effort is directed toward solution of problems, not the creation of new problems". Cavalieri was the only speaker to dwell at length on what he called "the hazards of success", suggesting that so far the discussion of recombinant DNA research has failed to consider the implications of the successful manipulation of the genetics of, for example, crop plants. The result, he said, "would be a change in the intricate balance of our environment, which has taken millions of years to establish".

Wald was the most outspoken critic of the research and the guidelines, however. He suggested that the recent outbreak of Legionnaires

Disease in Philadelphia has provided "a beautiful model" of what a public health problem with recombinant DNA research would be like—"unidentifiable and impossible to trace to its source". Wald argued that recombinant DNA research should not be conducted in universities, but it should be isolated in one or two national safety facilities.

As for proponents of the research, the most outspoken was James Watson, Director of the Cold Spring Harbor Laboratory. Watson said that he once told former Presidential candidate Sargent Shriver that the furore over recombinant DNA research is "the most overblown thing to enter the American scene since (President Kennedy) created the fallout shelter". Watson argued he could see no danger in transplanting genes from higher organisms into viruses or bacteria, and he said that he could see little hazard even in transferring genes from viruses known to cause cancer in animals into bacteria. The impact of infection by such organisms would be "negligible" compared with the impact of infection by the viruses themselves, he said, and suggested that "the marginal danger of this thing is a joke compared with other dangers". He suggested that the Attorney General's office could better spend its time looking into the potential health hazards of hair dyes and flame retardants in children's clothing. "What started as an attempt by the scientific community to be responsible takes on the aspects increasingly of a black comedy", Watson stated.

Faced with such widely conflicting viewpoints, the New York Attorney General's office is going to have a tough time deciding whether or not to impose additional controls on researchers in New York. Its decision will, however, be carefully watched in other state capitals. □

● Eleanor Lawrence adds from London:

The US National Institutes of Health guidelines and the report of the British Williams Committee have now been followed by recommendations for the conduct of *in vitro* recombinant DNA research by two European organisations, the European Science Foundation (ESF) and the European Molecular Biology Organisation (EMBO).

The ESF makes a number of suggestions. It says national registries of recombinant DNA research, analogous to the Genetic Manipulation Advisory Group proposed for Britain, should be set up in each country. It also suggests that all industrial, university and government laboratories be legally obliged to declare relevant aspects of

their work to such a registry. The supervisory and monitoring machinery would remain a national responsibility. There is a further recommendation that the code of practice drawn up by the Williams committee be followed rather than the NIH code since Williams specifies a greater degree of physical containment for most experiments, and is more flexible.

The EMBO guidelines also support the idea of national advisory groups, which would specify the conditions of containment to be used. EMBO says that either the Williams or the NIH codes of practice may be followed, but not a mixture of both. The different degrees of emphasis on biological and physical containment in the two codes, it contends, could lead to confusion.

Both organisations emphasise the need for regulations to operate at the same level in different countries and

therefore suggest that the national advisory committees should meet regularly. In addition they both propose that suitable experiments should be undertaken to assess the conjectural risks associated with recombinant DNAs and to pave the way towards eventual adjustments to the guidelines.

In Britain, however, many biologists are unhappy about the draft Health and Safety Commission (HSC) regulations now being circulated for comment. The regulations require among other things compulsory notification to the HSC of any experiment intended "to alter or likely to alter the constitution of any microorganism". Although the HSC has asked which experiments should be excluded, this type of blanket regulation, and the fear that proposals submitted to the HSC would be delayed interminably by bureaucratic machinery, have stimulated a group of

scientists to ask government that the HSC regulations should be withdrawn completely. They wish to see the voluntary procedures recommended by Williams followed instead.

There is strong feeling that in this instance the HSC is not the most appropriate body to deal with the complex scientific issues involved and that public interest would be better served if they were considered by an expert committee such as the Genetic Advisory Manipulation Group for example. The Health and Safety Commission would still have powers under the present Health and Safety at Work Act to inspect laboratories to see whether the containment procedures recommended by Williams were being implemented, and could probably be brought in by GMAG to prevent experiments considered unsafe being carried out. □

COMECON

● Scientific cooperation between Comecon countries is still "incommensurable" with the requirements and potentialities of the member countries, according to the Polish journal *Sprawy Międzynarodowe*. The relatively slow development of joint research and development projects is to a certain degree attributed to "subjective factors" such as lack of personal initiative, but also to the "excessive autarchic tendencies" of individual bodies which often necessitate intervention by the "central authorities" (that is, Party and Government) of the member countries.

A number of remedies are proposed to help the integration of research which is a fundamental tenet of Comecon planning. Most of these relate to improvements in organisation and management, the integration of science and industry and so on. One, however, suggests a basic lack in the whole policy of cooperation. This involves the question of funding. At present, it appears, the necessary funds are obtained often only after protracted negotiations between the countries concerned. To speed up the process, it is suggested that a special fund should be created within the International Bank (in Moscow) for the integration of research and development in the Comecon countries, with, in due course, the possible establishment of a Comecon bank especially for the financing of research.

● Since the discovery of lasers, laser research has been seen as a modern and progressive subject, eminently suitable for Socialist countries. The Second International Conference on

Luminescence at Szeged in Hungary last month showed that interest continues unabated. In particular, a Hungarian team described the use of lasers to determine "hitherto-undiscovered properties of substances", while Soviet, Polish and East German specialists reported considerable success in the use of lasers to investigate photosynthesis.



● Cosmonauts from all the Comecon countries—including Cuba and Mongolia—are to take part in manned space-flights between 1978 and 1983 as part of the Interkosmos programme. Until now, the programme has consisted solely of the launching of unmanned satellites, put into orbit by Soviet rockets and carrying experiments and equipment provided mainly by the Soviet Union with some participation from the European members of Comecon, notably East Germany and Czechoslovakia. Not surprisingly, Soviet participation in the new programme will also be considerable: as well as providing the spacecraft, orbiting stations, and launch facilities,

the Soviet Union will train the cosmonauts—training will take place at the Soviet "Yuri Gagarin" training centre. And the wording of a recent TASS communique suggests that at least one crew member in each flight will be from the Soviet Union.

● *Chemicisation*, the massive, widespread, undesirable use of artificial fertilisers, has long been a cornerstone of Soviet agricultural policy, and, not surprisingly, has become standard practice in other Comecon countries. In certain cases, however, the results have not been entirely successful. In Hungary, for example, excessive use of fertilisers has considerably reduced the sugar content of sugar beet in recent years, so that while the yield of beet itself has increased steadily, the overall sugar yield has remained constant. To encourage the production of maximum-yield crops rather than overall gross bulk, the Director-General of the Hungarian Sugar industry recently announced a new scheme of payment for sugar-beet crops under which farmers will receive a premium based on sugar content. The Hungarian experience is, apparently, not unique; recent Soviet resolutions on the further improvement of agriculture stress the need "to increase research on the study of the effect of mineral fertilisers and other chemical products on the quality and biological total value of agricultural production". This marks a radical change from traditional attitudes: until now the largest crops rather than the best have always won official acclaim.

Vera Rich

Same song, different meaning

Chris Sherwell reports on the latest developments on the European Community's research front

THE EEC's Council of Research Ministers did their turn again at the end of last week when they met in Luxembourg. In most respects it was a repeat performance of a long-running drama, but it did have the added merit of a new act to take it further towards the worthy ending it has so far lacked.

The Ministers were unable to make the crucial and much-delayed decision on the siting of the Joint European Torus (JET), the Community's sorely afflicted fusion project. It was that which made the meeting a repeat performance. But earlier expectations of progress on this particular matter had begun to die anyway, principally because the whole issue has in recent months become inextricably intertwined with the Community's four-year joint research programme, now under discussion.

When the Ministers left Luxembourg they had at least resolved a good deal concerning JET apart from the site. They were able to agree certain details regarding financial arrangements (though not others), as well as the project's legal status. It was these accords which helped mainly to redeem the show, but until the site is decided they can have little real practical meaning.

The meeting agreed that the European Commission will put up 80% of the costs, with the host country bearing 10% and the other participating countries the remaining 10%. The host country will also meet the costs associated with supplying the site with basics and with dismantling the project on completion in several years' time. At that point the equipment will be the host country's property. The Ministers further agreed that the project was a "joint enterprise", that is, independent of the host country and a Community project to be identified as such.

They were, however, unable to decide on staff numbers, although there was agreement that the host country would second people to the project and that others would be engaged as temporary agents of the Commission. Outstanding matters will be discussed by the member states' permanent representatives in Brussels, as will another issue, the precise costs involved. Since the Commission contribution of 108 million units of account (at March 1975 prices) was agreed, costs have risen and the project is being talked of in terms

of a four-year construction effort rather than five years. Some modification may therefore be necessary.

Much of this was largely in line with Commission proposals made a couple of weeks earlier. Regarding the site, however, the Commission was in favour of the Joint Research Centre (JRC) at Ispra in Italy—itsself nothing new—but had made a new proposal should no Council decision be reached before the end of the year. In that event, it suggested, the matter should be passed over to the Commission itself, which would then decide on the basis of an opinion from its own JET committee—which decided matters by a two-thirds majority.

This, a precedent which did not look like attracting many Ministers, was not discussed in Luxembourg. Another complication had already crept in some weeks earlier, when it was agreed to discuss JET in the context of the four-year joint research programme. Although designed to hasten agreement on the JET site, it introduced a bargaining element well suited to Community *realpolitik*. With details of the research programme themselves matter of dispute, movement on JET became contingent upon movement on the research programme.

At last week's meeting demands concerning the programme came, perhaps ironically, from three of the four main contenders for JET—Britain, France and Germany. Britain's starting position, on which it is flexible, was to seek reductions in staff of about 20% and cuts in certain expenditures amounting to £15 million at the Community's four research centres. Italy argued that this would threaten the centres, especially Ispra, the fourth JET contender. No one is saying that Italy and the Commission are now preparing for the worst, but there is speculation that if JET isn't to go to Ispra only a large alternative research programme—perhaps the one proposed, intact—would provide sufficient compensation.

Certainly the British were keen to emphasise that the site decision ought to be taken on scientific and technical grounds only. That would help the Culham site's case, but there would still be the matter of the French and German sites of Cadarache and Garching. Of the two, Germany with its stronger economy is the stronger candidate, yet the opportunity to strengthen its case still further through a higher host country contribution was apparently allowed to pass. French

research faces similar problems to Britain's, but this may say more about the relative weakness of its candidacy than Britain's ability to take on the project. The latter's enthusiasm for the project going to Culham remains undiluted by the current crisis facing its research community and economy generally.

As the Luxembourg meeting broke up the recipe was there for a good deal of haggling between the parties between now and the next Research Ministers' meeting on November 18. The understandings reached on JET and the joint research programme are provisional until a decision on the JET site is reached. If that decision, already delayed a year, is not made then, it could pass directly to the Council of Prime Ministers. If they didn't agree and refused to leave the matter to others, the Commission could go on funding the project in the way it has done hitherto. The fate of the project, however, will be more uncertain than ever. □

● The European Science Foundation (ESF), members of which are national research councils and academies in Europe, is due to hold its general assembly in Strasbourg this week. The Foundation involves a total of 43 member organisations from sixteen European countries. The President of the Executive Council, which will present its second report to the assembly, is Sir Brian Flowers, the UK physicist.

The Foundation aims to encourage the emergence of a close-knit community of science and research in Europe; this is also a goal of the EEC proper, with which the ESF has links through the Director General for Research, Science and Education at the European Commission. The ESF endeavours to do this by providing a forum for the exchange of information and experience, particularly regarding the way the member organisations actually operate. It also seeks by this method to encourage activity aimed at producing joint European action in certain fields.

As an international non-governmental non-profit organisation it lacks the financial means to give effect to its advice, but it can and does examine specific areas and maintains contacts with other concerned organisations, like EMBO and ESA. The Executive Council reports further progress in such areas as astronomy, space science, genetic manipulation and mathematics, in all of which it had sought to take initiatives during its first year of existence.

IN BRIEF

SERI delay

Another three-month delay has been announced by the Energy Research and Development Administration (ERDA) in the timetable for establishing the Solar Energy Research Institute (SERI), the facility which may eventually become the focal point for solar energy research in the United States. ERDA was hoping to choose a contractor to operate SERI and a site for the facility in December, but it now says that a decision cannot be made before March.

The delay stems chiefly from the fact that there is fierce competition among states for the contract to operate SERI, which will bring considerable amounts of federal money, jobs and prestige. ERDA has received 20 proposals for the contract and so far has eliminated only one of them.

The three-month delay is only the latest in a series of setbacks to the plans for SERI, first approved by Congress in November 1974.

SGHWR report shortly

Last week the final witnesses gave evidence on Britain's choice of nuclear reactor to the House of Commons Select Committee on Science and Technology. The committee says it hopes to publish its report in about a month, having questioned the Energy Minister Mr Benn, the Central Electricity Generating Board and the South of Scotland Electricity Board, the UK Atomic Energy Authority and the National Nuclear Corporation, and the Health and Safety Executive and Electrical Power Engineers Association.

Since the choice of the British Steam-Generating Heavy Water Reactor two years ago it has undergone substantial modification (a reference design was only submitted in June of this year) and expenditure on it was deferred for a year in this summer's government spending cuts. Continuing doubts about whether it was the best choice made

the committee decide to look at the whole question afresh.

Protest over Nancekuke closure

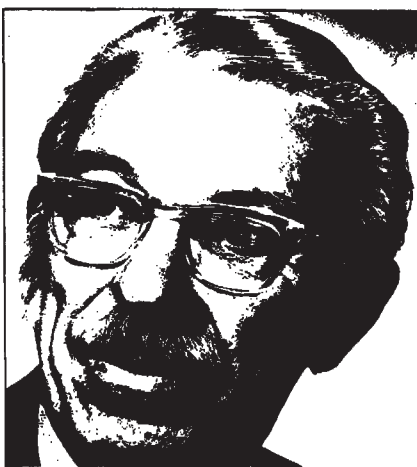
Civil service unions are protesting to the Secretary of State for Defence about the plans to close the Chemical Defence Processing Establishment at Nancekuke, Cornwall—due, according to the unions, in March 1978. Some of the work of the station will be moved to Porton Down in Wiltshire, another defence establishment.

The union objects on the grounds that Nancekuke's function is the production of defence chemicals, a job that Porton Down, a primarily research establishment, is not equipped to do. According to the 1973 Nugent report, closing the station would save around £80,000 a year, mainly in administrative costs, but the unions claim that money would not be saved.

LEGISLATION to control toxic chemicals was as inescapable as are the facts that all substances are chemicals, all chemicals (depending on the level) are toxic, and all human activities are the target of regulation. Passage of the new law was accelerated by some notable gaffes by industry. Some of these mistakes were inadvertent because they preceded recent advances in toxicological testing. In other cases there was a reluctance to adopt known procedures in testing industrial compounds; some of these had long been obligatory for proposed new agricultural chemicals and food additives.

Insecticides bore the brunt of the earlier demands for increased regulation, perhaps because the methods used in applying insecticides were often easily visible, as when residential areas were sprayed to control mosquitoes. Conversely, food additives got into trouble with the public partly by being honest. Their names are required on labels of foods in which they are put. Few scientists, and certainly no non-scientists, can derive a sense of epicurean satisfaction from contemplating the words "butylated hydroxytoluene" on the list of goodies in a prepared snack. Such nomenclature heightens the conviction that we are surrounded by a sea of new synthetic chemicals even though our species has been sniffing and enjoying the odours of allyl isothiocyanate and diacetyl for many generations.

The new law should bring protection to workers in chemical factories. Vinyl chloride, kepone and dichloromethyl ether furnished three examples of inexcusable incidents in which such workers were exposed

Curbing chemicals**THOMAS H. JUKES**

to toxic amounts of injurious chemicals. There is uncertainty and disagreement as to where the levels of such substances become tolerable, especially in the case of known or suspected carcinogens. However, it is known that the time of onset of cancer is lengthened by decreasing the dosage level, and lowering the dosage also decreases the probability that

cancer will actually occur. These relationships apply regardless of whether or not an actual threshold can be established.

When the pressures for bans are examined and compared there are some interesting paradoxes. Polychlorinated biphenyls (PCBs) escaped scot-free for about eight years after it had been shown that they were environmental contaminants on a global scale, toxic at low levels to many vertebrates, and distributed without control. Their American manufacturer made small gestures such as taking PCBs out of "carbonless carbon paper". Their use continued in transformers and capacitors, which furnish, among other things, a comfortable living for the middle classes. But the fury of middle-class environmentalists descended on DDT, the use of which was primarily needed to save lives and prevent disease in disadvantaged countries. PCBs are far more stable than DDT and are known to be injurious to human beings as contaminants of cooking oil. Fortunately PCBs are on their way out, after being used as examples for the need of the new bill.

Let us hope that the aroused consciousness of possible injury from "new chemicals" will eventually result in a balanced study of hazards from both synthetic and "natural" compounds. In the meantime I am trying to avoid a feeling that I am endangering myself when I sniff the smoke of a camp fire.

correspondence

Exotic viruses

SIR,—In last week's article on exotic viruses (page 625), an error was inadvertently introduced during the editorial work.

The identification of a Marburg-like agent announced two weeks ago was from material obtained from patients in a recent outbreak of illness in Zaire and the south of Sudan only.

ARIE ZUCKERMAN

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Engineers' action

SIR,—In your Editorial of October 14 on the Institution of Chemical Engineers' report on Materials and Energy Resources you take that body to task for attempting "both to stimulate reasoned discussion on crucial resource problems" and trying "to get something done about them." But the professional engineer's job is precisely "to get things done", not simply to establish the truth; and planning ahead is part of his work. So, when a body of engineers, having closely surveyed a field in which they have specialised knowledge and competence, foresee a dangerous situation developing, it is their duty not merely to point this out but to make recommendations for action—in this case by the government. And if not they—who else?

You suggest, too, that in such long-term matters "the Working Party might have done better to look more closely for international solutions". But practically-minded engineers are more humbly concerned with what can be done here and now, even when

their sights are on the future; and their chances of influencing government thinking, upon which you set such a low value, are at least a great deal higher than the likelihood of setting the world to rights.

P. M. C. LACEY

University of Exeter,
Exeter, UK

Whale resources

SIR,—Dr Robert May (Sept. 9, page 91) gave an interesting commentary on the problems of managing whale resources vis-à-vis other items in the relative values of the discount rate and the potential rate of increase of the resource is stressed. In this comparison the discount rate can be defined as δ where the present value of a whale caught in x years time is $e^{-\delta x}$ times the value of a whale caught today. May implies that δ is approximately equal to 'a', the interest to be expected from a deposit in a bank (10–20%).

In an age of inflation, and changing values of products from limited natural resources vis-à-vis other items in the economy, this may be misleading. The value in pounds of a whale at the time it is caught in x years time can be expressed as $e^{(b+c)x}$ times the present value, where b is the general inflation rate and c the rate at which the net value of products from a whale has changed differentially relative to average values of all commodities. b is positive and c may also be if prices for meat and other products from whales increase faster, and/or the price of fuel and other resources used in catching whales increase slower than prices in general.

The value of the discount rate to be used in determining the economic attractiveness (net present value) of a

whale caught later rather than now is given by $\delta = a - b - c$, which may not greatly exceed and may even be less than the rate of increase of whale stocks of around 5% annually. That is, to the extent that δ is less than a , the economic self-interests of a well-informed sole owner of a whale resource will conflict less seriously with conservation policies than implied by May.

The more important reasons for poor whale management have been the lack of an owner and poor information. While whales are common property, the individual whaling enterprise (company or even country) can ascribe little value to a whale in the future since there is no guarantee that it will be able to harvest that whale or its offspring. Also in the absence of well-funded research, whaling interests have been poorly informed of the longer term implications of management proposals. In the resulting fog of uncertainty about the future, their interests have naturally focused on the present and more clearly visible events. Both these effects have resulted in undue emphasis being given to maintaining current catches at a high level.

On another point, it seems unlikely, given the low (less than 10% per annum) rates of mortality and reproduction of whales that the unexpected changes in catch rates of fin whales mentioned by May is really indicative of population instability. It seems much more likely that with the small number of whaling expeditions now operating, the catch per unit effort is a poor and highly variable estimator of the abundance of whales in any particular area in a single season.

J. A. GULLAND

Department of Fisheries,
FAO, Rome



Competition 10

Very Many Hairy Little Pigs Live In The Torrid Argentine=valine, methionine, histidine, leucine, proline, lysine, isoleucine, threonine, tryptophan,

arginine (the amino acids essential to the rat). £10 for the best mnemonic in any field; entries by December 1 please.

Competition 9 asked for new words to describe some existing or future scientific concept or phenomenon. Entries galore: the winner, D. R. Reed of The Hague, The Netherlands, submitted six definitions, four of which we thought particularly successful: *cannobolism*—extreme form of competitive behaviour for scientific awards; *fornacation*—implantation of genetic (RNA based)

information in human ova; *multiplication*—means of obtaining the product of two numbers without the use of remote microprocessors; and *plutophagy*—simultaneous solution of world population and radioactive pollution problems.

Honourable mention for R. A. Davis of Epsom, Surrey, with "*qualms*—any new elementary particles, in search of a name"; and Allan N. Zacher III of the University of Missouri, with "*vitrogen*—an adjective describing a substance produced in a cell-free preparation".

news and views

Destruction of stratospheric ozone?

from Michael A. A. Clyne

STRATOSPHERIC ozone will eventually be depleted significantly if releases of chlorofluoromethanes (CFM) continue at the present rates. Legislation is not necessary now, but if after no more than two years, existing evidence still supports significant ozone depletion, action to restrict manufacture should be taken by governments. These are among the main recommendations of the Committee on Impacts of Stratospheric Change, (*Halocarbons: Environmental effects of chlorofluoromethane release*, National Academy of Sciences, Washington DC, 1976) set up by the US National Academy of Sciences.

In 1974 I reviewed Molina and Rowland's article (*Nature*, **249**, 810) in the same issue of *Nature*, on the effect of chlorofluoromethanes on stratospheric ozone. Then, neither I nor most other scientists, believed that the hypothesis presented by Molina and Rowland, and independently by Stolarki and Cicerone (*Canad. J. Chem.*, **52**, 1610; 1974) would in fact develop into one of the most important recent issues in environmental pollution. Briefly, this theory, which has now largely been supported by an authoritative report of the US National Academy of Sciences (*Halocarbons: Effects on stratospheric ozone, Panel on Atmospheric Chemistry*, National Academy of Sciences, Washington DC, 1976), is as follows. CFM 11 (CFCl₃) and 12 (CF₂Cl₂), which contain chlorine, fluorine and carbon only, are produced in large amounts in all developed countries of the world. Approximately half of the total output is used as propellants in aerosol cans for

personal consumer uses (such as hair-sprays and deodorants). Because of the stability that makes these compounds suitable for aerosol cans and for other uses, CFM 11 and 12 are very inert and no known major sinks for these compounds have yet been identified in the lower atmosphere (troposphere). Hence a large fraction of all past production of CFM 11 and 12, whether used for aerosols, car air conditioners or refrigerators is still present in the troposphere and indeed, these compounds will continue to accumulate there. Diffusion to higher altitudes occurs (Fig. 1), up to the stratosphere where most of the Earth's ozone (O₃) shield is located. The CFM can be photochemically dissociated there by far ultraviolet light at wavelengths around 190 nm. This light is not completely removed at stratospheric altitudes by the ozone and oxygen that normally filters out ultraviolet light, at wavelengths less than 300 nm, from reaching the Earth's surface. Photochemical action releases chlorine atoms from the CFM; and chlorine atoms react rapidly with ozone, giving chlorine monoxide (ClO) free radicals: $\text{Cl} + \text{O}_3 \rightarrow \text{ClO} + \text{O}_2$. . (1). The reaction (1) is then followed at once by the rapid reaction (2) of ClO with the large quantities of the free oxygen atoms present at stratospheric altitudes between 25 and 50 km: $\text{ClO} + \text{O} \rightarrow \text{Cl} + \text{O}_2$ (2). Chlorine atoms are thus recycled, and are able to consume more ozone; this sequence is termed a chain reaction. Finally, the cycle is terminated by removal of chlorine atoms in their

reaction with methane



Most of the HCl formed in reaction (3) is not recycled back to active chlorine. Although many other reactions are involved, some of which are mentioned below, the overall effect of CFM is thus clearly to deplete stratospheric ozone. The NAS reports conclude that this description of the problem, essentially as advanced by Molina and Rowland, cannot be seriously questioned, although there are new factors that probably somewhat diminish the effect on ozone depletion that they calculated.

There is no justification for the total rejection of an effect of CFM on ozone depletion, which had been maintained earlier by a few scientists. In their report, the NAS Committee state that "Selective regulation of CFM uses and releases is almost certain to be necessary at some time and to some degree of completeness". In reaching their conclusions, the NAS were guided by a panel of distinguished experts including one Briton, Brian Thrush, and one German, Dieter Ehhalt. The report is an impressive document, containing a large amount of carefully sifted information that will be invaluable to future workers in atmospheric sciences. The approach is low-key, thoughtful and critical. The evidence accumulated by the panel is a formidable case in favour of an effect of halocarbons on stratospheric ozone. However, it is considered that the risks of a delay of not more than two years in restricting CFM releases are low. Although most scientists will probably agree with these views, some will doubtless point to the admitted wide margin of error in the estimate of a 7% eventual ozone depletion, with CFM 11 and 12 releases at the 1973 level. (According to the Panel, the limits of 2 to 20% ozone depletion have a 95% confidence level).

Some will say that the risk is slight, and that in any case industry is already reducing production of CFM 11 and 12, and is actively seeking substitutes that will have a shorter tropospheric life, and hence be less harmful. It re-

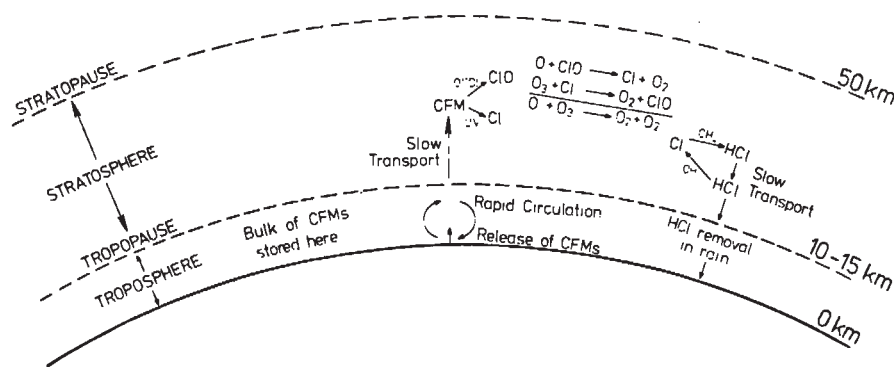


Fig. 1 The atmospheric ClO_x cycle.

mains to be seen whether the search for acceptable substitutes for CFM 11 and 12 is successful; this is not likely to be an easy problem. CFM 22 (CHF_2Cl), among others, has promise as a substitute for CFM 11 and 12 in spray cans.

Other people will maintain that the risk is unacceptable, and that legislation is needed now rather than (possibly) in two years time. They will point to the possibility that delay might lead to politicians and governments losing sight of a problem which is effectively postponed for two years, a time scale comparable to intervals between elections. However, it should be noted that as well as studies by US government authorities, both the EEC and the West German government are initiating substantial investigations into the CFM-ozone problem.

The British government published their report on chlorofluoromethanes and their effect on stratospheric ozone earlier in the summer. This report was prepared by the Department of the Environment (DOE) with considerable assistance and guidance from AERE, Harwell. Their conclusions are broadly in line with those of the NAS report, although they do not recommend any specific regulatory action by governments. The DOE report is a useful summary of results; although clearly the resources that are evident in the NAS report were not available to the DOE compilers. The referencing of the DOE report is rather random and non-specific, and some literature citations are an encouragement to whimsical day-dreaming! For instance: ICI (1976), Imperial Chemical Industries, private communication. Dubreuilh W. (1896), des hyper-keratoses circonscrites; *Ann. Dermatol. Syphilig.* 3rd Series, 7, 1158-1204; quoted by Blum (1956).

In order to predict the effects of CFM on stratospheric ozone, a wide variety of data are required. The scope and uncertainties of these data varies enormously. Some of the basic types of information needed are (1) direct measurements of Cl atom, ClO radical, and other concentrations in the stratosphere; (2) the reaction rates of key elementary chemical reactions, as determined in the laboratory, and applicable to stratospheric temperature ($\approx 220\text{ K}$); (3) knowledge of the transport of air masses from the troposphere to the stratosphere. The first type of data (1), is perhaps the most important, because direct measurement of concentrations is the final check that the correct information under categories (2) and (3) is being used. Unfortunately, data of type (1) are by far the most difficult to obtain and are extremely sparse. The only results (so far) are *in situ* measurements of Cl and ClO

How rigid the nucleotide?

from a Correspondent

CRYSTALLOGRAPHERS have become used to seeing every structural idiosyncrasy they unearth in the crystal structure analysis of molecules of biological interest become a peg upon which the edifice of some molecular biological theory is hung. This has been particularly true for the study of the nucleotide and amino acid monomers from which nucleic acids and proteins are constructed. Beginning with the use of the covalent bond lengths and angles observed in monomers in the design of molecular models of these biological polymers, the results of crystal structure determinations have been extended to provide constraints which might properly be assumed for hydrogen bond geometry, for the conformations which can be expected to be preferred about various single bonds and, for the patterns of arrangement of water molecules and ions around biological polymers. This fertile field has been exploited with ever increasing confidence and perhaps nowhere more than in the analysis of the relationship between the properties and structure of nucleic acids. An important development in this work has been the rigid nucleotide concept

which suggests that mononucleotides have a rather rigid conformation which is conserved when they are joined together to form a polymer. X-ray diffraction studies of nucleotides, nucleosides and crystalline polynucleotides have provided a great deal of evidence for this although a number of recent X-ray single crystal studies of nucleotides have suggested that the situation may well be rather more complicated than was at first thought. The need for caution in applying the rigid nucleotide concept too insensitively is further emphasised by Evans and Sarma in a recent issue of *Nature* (263, 567; 1976). From NMR studies they suggest that adenine-containing nucleotides and the oligomers derived from them are no more rigid than the nucleoside adenosine itself. These studies are of course of particular interest because they relate to the situation in solution. An important application of the results of this work which is considered by the authors lies in the analysis of the structure of yeast phenyl alanine tRNA determined by X-ray single crystal studies and in particular the correlation of nucleotide conformation and base-stacking.

in the stratosphere from two balloon flights carried out by J. G. Anderson (University of Michigan) in summer 1976 at Palestine, Texas. Both concentrations of Cl and ClO were determined by Cl atom atomic resonance fluorescence, which has been successfully used also for O atom and OH radical measurements in the stratosphere, by Anderson (*Geophys. Res. Lett.*, 2, 231; 1975; *Geophys. Res. Lett.*, 3, 165; 1976). ClO radicals were reacted with nitric oxide to give Cl atoms, which were detected by atomic resonance fluorescence. The technique was adapted from flow methods for laboratory measurements of Cl atoms and ClO radicals that were developed recently (see Bemand and Clyne, *J. Chem. Soc. Faraday II*, 71, 1132; 1975). Anderson's first Cl and ClO experiments have not so far led to satisfactory results, although the prospects for the atomic resonance and other methods look promising.

Additional direct verification is in principle obtainable from measurements of ultraviolet light intensity at ground level. Whilst such measurements have been made for a number of years, the natural variation of ultraviolet levels at the ground is large. The effects of CFM would probably only become observable in this way when

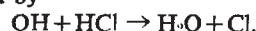
the ultraviolet level was already increased by a significant, probably unacceptable amount.

Data of type (2), the rates of chemical reactions, are considered carefully in the NAS panel report. One basic difficulty was to identify correctly the major reactions, of which there are a large number. The recently-proposed role of chlorine nitrate, ClONO_2 , has necessitated the introduction of several new reactions. The elementary reaction rates of chlorine nitrate are not, however, all well-defined. Fortunately, the model for ozone depletion is not very sensitive to these rates, and some confidence may be attached to the NAS panel's conclusion that the ozone depletion is reduced by a factor of about 1.7 by inclusion of chlorine nitrate in the model.

The NAS panel conclude that the largest uncertainty in the ozone depletion model is due to uncertainties in the relevant chemical reaction rate constants. They carefully identify the reactions introducing the greatest uncertainty as



followed by



These reactions are involved in the sink processes by which active chlorine

(Cl and ClO) is removed from the ClO_x cycle. Surprisingly, the two reactions of the ClO_x cycle, that is $\text{Cl} + \text{O}_3 \rightarrow \text{ClO} + \text{O}_2$ and $\text{O} + \text{ClO} \rightarrow \text{Cl} + \text{O}_2$, do not introduce large errors, because their rates have been carefully measured in the laboratory. The first measurements of $\text{Cl} + \text{O}_3$ and $\text{O} + \text{ClO}$ rates by Bemand, Clyne and Watson at Queen Mary College in 1973 (see *J. Chem. Soc., Faraday I*, **69**, 1356; 1973; Clyne and Watson, *J. Chem. Soc. Faraday I*, **70**, 2250; 1974) have been only slightly altered as a result of subsequent work in the USA and in this laboratory.

Significant uncertainty ($\pm$ a factor of 1.7) in the prediction of ozone depletion is also introduced by the one-dimensional model used by the NAS panel to approximate the distribution and transport of the chemical species involved. This model is equivalent to averaging the concentrations, motions and chemical reactions over latitude and longitude, leaving only their dependences upon altitude and time. This approximation is considered to be a

good one. For instance, the equivalents of averaged gas transport rates appear in the model as the vertical eddy mixing or transport coefficient, which depends only on the altitude.

One further very important question concerns the effects of an increased ultraviolet intensity (UV-B, 280 to 320 nm) at ground level on animals and plants. The biological effects of UV-B are not as extensively dealt with by the NAS reports as are other aspects. This may be due to the relative lack of knowledge in this area. It is clearly to be identified as a priority area for future work on the CFM-ozone problem. However, in general terms, the deleterious effects of UV-B radiation on living cells are well known. For example, there is a clear causal connection between the more common (but less serious) forms of skin cancer (basal and squamous cell carcinomas) and UV-B exposure. The connection between the less common, but often fatal malignant melanoma and UV-B, is suggestive but not certain, according to

the NAS report.

Release of pollutants other than CFM, which can also affect ozone levels, is another topic requiring extensive future work. Among halogen compounds, further work on methyl chloride formed by the burning of vegetation, is indicated. Also, Crutzen has suggested that methyl chloroform (CH_3CCl_3), whose manufacture as a degreasing agent is considerable and possibly increasing, may eventually cause ozone depletion comparable to that of CFM 11 and 12. Bromine compounds, released for example as methyl bromide in soil fumigants, are probably not produced in large enough quantities to have major effects. However, Br atoms and BrO enter into a similar, but more effective cycle to that of Cl and ClO.



The rate constants for the bromine reactions are not particularly well known. Nitrous oxide, apparently re-

THE *in vitro* cultivation of malaria parasites has been possible for many years but only one or a few successive cycles of development have been achieved and these successes have been mainly with the malaria parasites of monkeys. The amount of physiological and biochemical knowledge that has accumulated from such studies has been vast and it cannot be disputed that much of it is directly or indirectly applicable to chemotherapeutic and immunological studies aimed at the control of malaria. What is desperately required, however, is the long term continuous cultivation of human malaria parasites which cannot be maintained in standard laboratory animals. For some time now there have been rumours of the successful cultivation of *Plasmodium falciparum* in the United States and now two independent reports have appeared, giving the long-awaited experimental details (Trager and Jensen, *Science*, **193**, 673; 1976; and Haynes *et al.*, page 767 in this issue of *Nature*). There can now be no doubt that the continuous cultivation of *P. falciparum* can be achieved in simple media and that it should shortly be possible to obtain the yields of parasites necessary for the production of experimental vaccines.

Both media are remarkably simple. Trager and Jensen use a modification of an earlier medium (RPMI 1640 with HEPES buffer and serum) for the cultivation of *P. coatneyi* from monkeys (Moore *et al.*, *J. Am. med. Ass.*, **199**, 519; 1967) but substitute human serum for the monkey serum originally used. In essence, the medium

Cultivation of human malaria

from F. E. G. Cox

flows over a shallow bed of red blood cells which are disturbed as little as possible in an atmosphere of low O_2 and high CO_2 . The technical details are still being modified and Trager and Jensen began by using 7% CO_2 and 5% O_2 but changed this to 1% O_2 in later experiments with better results. The technique of Haynes *et al.* is more traditional, involving a supplemented medium 199 with 1% foetal bovine serum in an atmosphere of 3% CO_2 . No doubt many other ways will be found to grow *P. falciparum*, and Trager and Jensen report success using their medium in a candle jar (a container in which a candle has burned and extinguished).

The most important features of the cultivation techniques described seem to be the high levels of CO_2 and the fact that the number of red cells infected rarely rises above 1%, thus preventing the culture medium from becoming too acidic as a result of the metabolic activities of the parasites, which is a problem inherent in this kind of experiment. Although the multiplication of parasites recorded is low (a four-fold increase per cycle is assumed by Trager and Jensen) the numbers of parasites available for collection can be considerable if parallel cultures are set up and initiated from diluted cultures

at the end of each cycle. Haynes *et al.* obtained eleven successive cultures and Trager and Jensen more than twenty-five (and Trager has since reported continuous cultivation over 105 d, implying fifty or more cycles) so there is no reason why such cultures should not be maintained indefinitely.

The technique described by Trager and Jensen looks more promising than that of Haynes *et al.*, but the latter authors have made a major advance by showing that their cultures can be initiated from frozen blood samples. It should therefore be possible to initiate a culture and to freeze stabilates from that culture for the initiation of others. This would save the lives of countless owl monkeys (*Aotus trivirgatus*), which at the moment are the only sources of laboratory maintained *P. falciparum*, and also standardise experiments carried out in different laboratories.

The implications of these experiments are considerable, for as well as the biochemical and physiological observations which are now made feasible it should be possible to grow the numbers of merozoites (the stages that pass from one cell to another) needed for producing vaccines. The only homologous vaccine with real potential, in monkeys at least, is that based on merozoites (Mitchell, Butcher and Cohen, *Immunology*, **29**, 397; 1975) and the production of a suitable vaccine free from viruses is only possible from cultured parasites. With 96 million cases of malaria each year and with *P. falciparum* being the major killer the production and evaluation of such a vaccine should not be delayed.

leased by bacterial action on nitrogenous chemical fertilisers, is fairly stable in the troposphere. The possibility of this compound affecting stratospheric ozone levels should certainly be studied.

All in all, the ozone layer is fragile, and wide-ranging long-term studies of possible effects of pollutants not yet identified are essential. This should include the acquisition of more information on ozone reaction rates. Exclusive concentration on CFM 11 and 12 effects is unwise, since it is quite possible that other deleterious compounds may be present now, or at some future time, due to shifts in industrial production.

I have emphasised here the effects of CFM on the ozone layer and the consequential increase in UV-B radiation at the Earth's surface. It should also be noted that other climatic changes due to CFM cannot be excluded. For instance, Ramanathan (*Science*, **190**, 50; 1975) has stated that CFM 11 and 12 are sufficiently effective absorbers of infrared radiation (near 10 μm) to have a potentially significant effect on the Earth's heat balance.

The role of British science in the CFM-ozone problem is an important one. Much of the basic work leading to the original theories of Stolarski and Cicerone and Molina and Rowland, was carried out in the UK. Lovelock developed a sensitive electron-capture gas chromatograph, with which he was able to demonstrate the presence of various halocarbons arising from man's activities, and to obtain measurements of CFM 11 and 12 at various locations. The programme of work (COMESA) sponsored by the Department of Trade, and carried out partly in university laboratories and partly by the Meteorological Office on possible effects (slight) of Concorde on the stratosphere also

provided valuable input data for the CFM-ozone problem. Current initiatives in the CFM-ozone problem are being taken by industry; research on both CFM 11 and 12 effects, and on substitute CFMs is being carried out. Basic science relevant to stratospheric ozone is being supported by the Science Research Council, and recently the Natural Environment Research Council has announced its intention to concentrate more resources on atmospheric science. The financially small but scientifically significant support over many years in this area by the Royal Society through its Gassiot committee has also been very valuable.

With this expertise available, it is sad therefore that there is no sign at present that the British government is planning any major programme of investigation in stratospheric science related to the CFMs and ozone, although it does contribute through the EEC organisations to the European effort. No more than £100,000 would probably be allocated to such a new investigation, if mounted eventually by the Department of the Environment. Although the restraints of the present economic situation have to be acknowledged, it is a pity, nevertheless that it was not possible to use the experience gained in the COMESA research as the basis of an ongoing and centrally-planned programme of work on atmospheric pollution.

There is a message from all this for those who ask for more "relevant" research programmes, related to current needs of society. Without the undirected, basic research programmes which were carried out, the chances are that the risks to ozone from CFMs would not have been identified until much later, if at all. Identifying the problem is a large part of finding the solution. $\square$

sequences in pure form, by cloning in a bacterial host, has opened up the possibility of studying the sequences flanking the gene which may be important in regulation, and which cannot be obtained sufficiently pure for sequence studies by any other means. The sea urchin (*Psammechinus*) histone gene repeat unit has been cloned in phage lambda and some 25% of the nucleotide sequence determined by a group at the University of Zurich (Walter Schaffner).

The mammalian globin genes are also under determined attack in several laboratories. Charles Weissman (University of Zurich) and Richard A. Flavell (University of Amsterdam) reported the partial purification of the + and - strands respectively of rabbit globin DNA. Weissman and colleagues have isolated the + strand by hybridisation to immobilised globin cDNA; the - strand has been isolated by hybridisation with mercurated mRNA. The final purification step will be to hybridise the two strands, since only complementary sequences should anneal. This reconstituted DNA inserted into a plasmid should provide a source of the complete globin gene sequence including the flanking non-transcribed regions.

Still looking to the future, Bob Williamson (St Mary's Hospital Medical School, London) described a possible use for cloned globin gene sequences as chain-specific probes for the study of certain inherited defects such as the thalassaemias.

Recombinant DNA is also a powerful new tool for probing the immunoglobulin genes. As the first step to tracing changes in the Ig-gene coding sequences predicted by the theory of somatic mutation, Bernard Mach and colleagues (University of Geneva) have constructed plasmids carrying cDNA copies of a mouse myeloma light chain mRNA. The Geneva group has obtained plasmids containing constant and variable region sequences both separately and together. Hybridisation studies have shown that the untranslated region in kappa light chain messenger from different sources is highly conserved.

However, these experiments exemplify some of the conjectural risks which have caused concern about this type of work. The mRNA was not pure and indeed 40% of the initial recombinants contained unknown sequences. More worrying still is that myeloma cells are known to express C-type RNA viruses and that the plasmid carried a drug-resistance marker containing an IS sequence.

This problem is avoided in work from the Laboratory of Molecular Biology in Cambridge reported by Terry Rabbitts. Light chain mRNA

Nuts and bolts of genetic engineering

by Eleanor Lawrence

The Biochemical Society's 9th Harden Conference, entitled the Role of Recombinant DNA in Molecular Biology, was held at Wye College on September 20-24, 1976.

In the present state of the art, a meeting on *in vitro* recombinant DNA must of necessity concern itself more with means than ends. This is partly a result of the need to develop and refine a range of completely new techniques; but it is also a consequence of the almost unique situation in which the novel technology has been applied less

than whole-heartedly, as a result of the voluntary postponement of some experiments until experimental conditions minimising the hypothetical safety risks of this work had been agreed.

But now that experimental guidelines have been published in the United States and Britain, workers are preparing to clone genes from everything from maize to man. A foretaste of the power of these new techniques to investigate otherwise unapproachable problems of the organisation of the eukaryotic genome was given at the meeting.

The ability to obtain specific gene

from myeloma cells can be reverse transcribed into cDNA by starting with a hexanucleotide primer T₂G₃T that has been shown to prime only at a site just within the 3' end of the C region. In this way pure fragments containing the immunoglobulin V and C coding sequences only can be made, without the need for constructing plasmids by way of impure mRNA. A comparison of the reverse transcriptase "stutter" pattern of mRNA from a mouse T-cell lymphoma has produced evidence that in these T cells the immunoglobulin genes are at least transcribed.

The most elegant exploitation of the recombinant DNA technology was in work by David Hogness's group (Stanford University) on the organisation of the *Drosophila* chromosome. Taking advantage of the relatively small size of the *Drosophila* genome and the availability of polytene chromosomes, they have constructed plasmids containing different *Drosophila* gene sequences which have been mapped on the chromosomes. Hogness described analyses of the histone genes, the genes induced by 'heat shock', and of a sequence coding for an abundant mRNA. It is particularly intriguing that this mRNA sequence is found by *in situ* hybridisation to be located at 33 sites dispersed throughout the genome. What the dispersed gene sequence means in terms of evolution and control is still a matter for dispute. Hogness tends to the view that each individual sequence will be expressed in a different cell type, which would explain the maintenance of 33 separate sequences coding for the same product.

For those who wish to study the functional expression of eukaryotic genes, cloning in plasmids or phage has one overwhelming disadvantage. So far there are only a few reports that eukaryotic genes can be expressed in bacteria. Also, there will eventually be a need to put characterised, specifically mutated genes back into eukaryotic cells to study their expression. This might be done using animal tumour viruses that can integrate into mammalian cell chromosomes.

The potential of SV40 as an 'eukaryotic vector' is at present being explored and Paul Berg (Stanford University) described the construction of an SV40 vector in which the late genes have been deleted, and its use to clone fragments of lambda DNA in monkey cells. Additional vectors are under construction in which the early region has been deleted; these may more readily allow transcription of the inserted DNA. All these SV40 vectors are dependent on a temperature-sensitive helper virus for growth.

In this system there is however an

ultimate constraint on the size of the inserted DNA because of packaging into SV40 protein coats of defined size. This problem may be overcome in a system that Joe Sambrook (Cold Spring Harbor Laboratory) is developing. He has found that human cells transformed by SV40 virus or DNA contain a large number of free SV40 DNA molecules. Transformation rather than plaque formation could be used as the selective marker for recombinants, and since no helper virus is needed and no infectious virus is released less rigorous containment conditions are required.

A system in which cloned eukaryotic DNA might be transcribed and translated was described by Janet Mertz (Laboratory of Molecular Biology, Cambridge). Both prokaryotic and eukaryotic DNA injected into the nucleus of *Xenopus* oocytes appear to be reconstituted into chromatin and transcribed but whether the transcripts are sensible or are translated is still problematical. One use for this system would be in identifying eukaryotic promoters by fragmenting well-characterised genes and then determining which pieces are transcribed.

So far most attention has been focused on the use of restriction enzymes for mapping gene sequences and producing recombinant DNAs. But there is another side to the story as pointed out by Adrian Bird (MRC Mammalian Genome Unit, Edinburgh) who has been studying the natural methylation of some restriction targets, in the ribosomal DNA of *Xenopus laevis*, which renders them resistant to attack. By analysis with a set of restriction enzymes he has shown that the probability of methylation at each suitable site is high (0.99) but that there is a unique site within the rDNA repeating unit that remains unmethylated in up to half of repeats. He has also shown that the pattern of methylation is copied onto newly synthesised DNA *in vivo* and so is inherited by a daughter somatic cell from its parent.

The prospect of using eukaryotic genes cloned in bacterial cells to make large amounts of valuable gene products is still very much in the future. But Noreen Murray (University of Edinburgh) described ways of harnessing the enormously efficient lambda leftward promoter to provide enhanced expression of inserted bacterial genes, a technique which could be applied to produce large amounts of many useful phage and bacterial enzymes.

Although the issue of containment levels and possible safety risks hardly surfaced officially, safer experimental procedures were much on everyone's mind. Paul Berg expressed concern that antibiotic resistance was still so widely used as a selective marker since

it could contribute to the persistence of any escaped vector, not only in the human intestinal tract but in the general environment (Sydney Brenner has been quoted as remarking that the world is a dilute solution of tetracycline). The proliferation of different vectors for cloning recombinant DNA also led Berg to propose a register clearly setting out their desirable and undesirable features (such as the possession of insertion sequences). Berg said that he would collate a list, from names and addresses sent to him, of people working with vectors. This would be published in *Nucleic Acid Recombinant Scientific Memoranda*, produced by and available from the National Institutes of Health. □

Actins plural

from Dennis Bray

The impersonal air that is usual in a comment like this wears thin when its author is forced to eat his words. For it was indeed I who solemnly predicted, three years ago, that the actin present in different kinds of cells was one and the same gene product (Bray, *Cold Spring Harbor Symp. quant. Biol.*, **37**, 567; 1973); and in this I could not have been more wrong. Hardly were the words out of my mouth when new forms of actin began to spring up like mushrooms. So well they grew, and in such numbers, that little remains now but to turn to Philosophy and learn the value of the refutation of a cherished hypothesis. (Popper, *The Logic of Scientific Discovery*, Hutchinson, 1959).

It has always been clear that the actin and myosin of non-muscle cells must behave in a different fashion to that of muscle: the exigencies of motility and cell division ensure that. But evidence for differences in amino-acid sequence, rather than in the modifying influence of ancillary components, is harder to come by. Some early hints exist in the literature, such as that in Fig. 12 of a paper on the actomyosin from brain (Berl and Puszkin, *Biochemistry*, **9**, 2058; 1970). This shows that brain actin and muscle actin run differently on urea-acrylamide gels and seems to have been overlooked in recent papers that present the same fact (Storti and Rich, 1976, *Proc. natn. Acad. Sci. U.S.A.*, **73**, 2321; 1976; Storti, Coen and Rich, *Cell*, **8**, 521; 1976). But it was probably in the course of a thorough examination of platelet actin, that the first unambiguous differences—in the form of cyanogen bromide peptides—were found (Booyse, Hoveke, and Rafelson, *J. biol. Chem.*, **248**, 4083; 1973). A later comparison of peptides from brain actin to those from muscle also showed differences (Gruenstein and Rich, *Biochem.*

biophys. Res. Commun., **64**, 472; 1975).

At this point it was still possible, with difficulty, to believe in one actin. The cyanogen bromide evidence was clear, but perhaps there had been an incomplete cleavage. Criticism of the work on brain actin was possible, since the data showed as much variation between different samples of muscle actin as between muscle and brain. But there was little point in chopping these actins down: no sooner the attempt than two stood where previously there had been but one! (Dukas, *The Sorcerer's Apprentice*, 1897). Evidence was adduced, first for platelets and then for other cells, that not only was their actin distinct, but it was multiple. The platelet actin that Booyse and his collaborators studied was extracted as if it was muscle actin, and it obligingly behaved in this way. But this was only a small fraction of the total actin and when a different approach was tried, a protein with a quite different ability to polymerise was obtained. (Abramowitz, Stracher, and Detwiler, *Arch. Biochem. Biophys.*, **167**, 230; 1975). Moreover amoeba, fibroblasts and brain, also give small amounts of polymerisable actin, or large amounts of unpolymerisable actin, according to the extraction used. There is still a lurking question of whether a slight denaturation is involved, but on the face of it this is evidence for two actins.

An even greater virtuosity is apparent in developing muscle which shows no less than three actins (Whalen, Butler-Browne, and Gros, *Proc. natn. Acad. Sci. U.S.A.*, **73**, 2018; 1976). The evidence here is from gels lacking SDS—and it must be recalled that in some conditions even pure muscle actin will split into a number of bands (Pardee and Bamberg, *J. Neurochem.*, **26**, 1093; 1963; Carsten and Mommaerts, *Biochemistry*, **2**, 28; 1963)—but the changes that accompany differentiation are convincing. Finally, lest there are any lingering doubts about the reality of the differences considered, comes definitive evidence from the amino acid sequencers: slow, but exceedingly hard to demolish. With the complete sequence of muscle actin in hand, Elzinga and colleagues have now compared peptides from human heart and human platelet actin (Elzinga, Maron, and Adelstein, *Science*, **191**, 94; 1976). A diminutive difference exists—1 residue in 29 is changed, from a harmless valine to an innocuous threonine—but it is enough.

From the wider biological standpoint these results add to other examples in which closely related proteins are used to perform similar, but distinct, functions. The evidence available for tubulin suggests that the various organelles in which this protein appears are based on different gene products;

and a similar statement probably holds for myosin and tropomyosin. Undoubtedly there are advantages in this: the process of gene duplication is familiar in haemoglobin, and provides a ready-made protein on which evolution can go to work. But, if Cassandra is allowed one parting shot, in the matter of the disposition of these proteins within the cell many mysteries remain. It seems scarcely credible that such similar molecules could be moved within the same small space and yet not cross-react or finish in the wrong place.

Southampton model fair

from N. MacDonald

A research conference on nonlinear mathematical modelling was held at Southampton University on September 13–17, 1976. It was sponsored by the Social Science Research Council and organised by D. R. J. Chillingworth, H. B. Griffiths and D. A. Rand of the mathematics department at Southampton.

From the viewpoint of a physicist working on non-linear modelling in biology, the most satisfying talks dealt with specific problems from the hard sciences, handled with mathematical sophistication. There has been much interest recently in the behaviour of travelling waves in solutions undergoing oscillatory chemical reactions, such as the Zhabotinskii reaction, both as objects of study in themselves and as a possible paradigm for pattern formation in organisms. N. Kopell (Northeastern University, Boston) described two elegant applications of bifurcation theory, to the outward propagation of chemical waves from a centre, and to the collision of two such waves.

A considerably deeper physical problem was the subject of a fascinating lecture by G. Jona-Lasinio (University of Rome). He described recent work by the Russian mathematicians Bleher and Sinai on phase transitions (*Commun. Math. Phys.*, **45**, 247; 1976; see also a review by Jona-Lasinio in *Nuovo Cimento*, **26B**, 99; 1976). These are difficult phenomena to model because one has to build up from local assumptions about interactions between atoms to an essentially collective large scale switch in the behaviour of the material. Bleher and Sinai treat this scaling process as an iterative operation on the probability distribution. The asymptotic large scale forms of the probability distribution are obtained as the fixed points of a discrete dynamical system on a function space, and phase

transitions are related to the bifurcations of these fixed points.

The bifurcation theory of discrete dynamical systems is a very popular subject at present. The great richness of the behaviour of solutions of even the simplest difference equations (discrete dynamical systems on the real numbers) was the subject of a recent review by R. M. May (*Nature*, **261**, 459; 1976). At this meeting J. Guckenheimer (University of Santa Cruz) described the application of this kind of mathematics to a model of the population fluctuations in the famous blowfly experiment of Nicholson.

Turning to the more qualitative surveys, B. Goodwin (University of Sussex) discussed morphogenesis, in particular recent results on the circumferential field in the development of the insect limb. He stressed the gap between the field concept, which is effective in coordinating data, and the underlying mechanisms, as well as that between the abstract dynamics suggested by catastrophe theory and the observable expression of form in the organism. Evidence that the conceptual difficulties involved in understanding complex biological systems are of a different order from the mathematical difficulties of current modelling techniques, came in the lecture by R. Rosen (Dalhousie University).

Several of the lectures on the social sciences had some common themes. A. C. Renfrew (Southampton University) dealt with attempts to set up a framework in which questions about the growth and decline of cultures can be handled by dynamical modelling techniques, analogous to those of the Forrester–Meadows group. K. L. Cooke (Pomona University, California) described the application of one such method to Renfrew's description of the Aegean culture. A. G. Wilson (University of Leeds) gave an account of the great variety of modelling techniques that have been applied to urban geography, and stressed the need to move on to a more integrated approach.

P. J. Harrison (University of Warwick) discussed the problem of short term prediction in economic and social contexts. Here standard methods apply essentially to linear behaviour, while the need to handle non-linearity becomes apparent especially when concerned with the need to keep options open for the possible reversal of an existing policy.

Although the main aim of the meeting was to set up an interaction between pure mathematicians and modellers, it was evident that cross-disciplinary contacts between modellers were equally stimulating. These were mainly informal, although some structure was given by a series of meetings in the archaeology department. All

open-minded members of the conference must have taken away, as well as their copies of Poston and Stewart's little red book, and the good resolution to think more geometrically, much benefit from the chance to talk with people from a remarkably wide range of disciplines. □

Venus is another differentiated planet

from G. Fielder

MANY scientists were surprised to find that the Moon was a differentiated planet. Now, *in situ* measurements of parts of the surface layers of Venus have indicated that the Earth's "sister" planet is more like the Earth than the Moon in its radioactive element content. Whereas measurements of the potassium, uranium, and thorium concentrations made in October 1975 using Veneras 9 and 10 indicated the presence of basaltic type rocks, earlier results from Venera 8 (July 1972) had indicated the presence elsewhere of rocks similar in their radioactivity to that of a typical terrestrial granite.

Writing with K. P. Florenskii, A. T. Bazilevskii and A. S. Selivanov in *Doklady Akad. Nauk. SSSR* (228, 570; 1976) the late A. N. Vinogradov has reported studies of the photographic panoramas and other data transmitted from Veneras 9 and 10 by way of a relay satellite. Telecameras mounted 1 m above the surface took high Sun photographs, including the one reconstructed in Fig. 1, with a resolution of about 1/3 degree. So effective was the

dense atmosphere of Venus in diffusing the weak light at ground level that high contrast versions of the photographs show "artificial shadows" which always point towards the appropriate camera.

The upper right hand corner of Fig. 1 shows the skyline, estimated to be several tens of metres distant. The sharpness of the skyline indicates a fairly high atmospheric transparency and, consequently, a comparatively low dust content. If any wind had been blowing across the surface when the pictorial data were transmitted, the wind had not been dust laden. Nevertheless, the authors have suggested that the clod-like nature of the dark soil filling the spaces between the numerous rock blocks may have resulted, in part, from the evacuation of fine particles by a weak wind. An alternative explanation, they suggest, is that the clods were produced following hydration of the particles.

Typically, the rock blocks themselves have dimensions of some tens of centimetres. Some are angular, others rounded. Many indicate that they have been detached from hard, layered bedrock. Others, which appear to be mostly buried, could even be outcrops of bedrock. A few rocks have cracks which cross the bedding plane. Others appear to be vesicular.

Together with the knowledge from radio observations that the surface layers of Venus are underdense, the picture data establish that certain dynamic processes are in operation there now. If the corrosive agents hydrochloric acid and hydrofluoric acid observed in the upper atmosphere of Venus are present at ground level they could be an important factor in the rounding and disintegration of the

rocks to generate the soil. Whatever the predominant mechanism of denudation on Venus, it is clear from the abundance of sharply sculptured rocks on the surface where Venera 9 landed that this is a geologically young area of the planet.

The clouds of Venus, which prevent our viewing its surface directly, are found at up to 70 km above the surface. Most meteoroids of the present day populations would probably disintegrate in the thick, dense atmosphere (97% CO₂) before reaching the surface so that recent disturbances of the rocks of Venus are likely to be of internal origin. Now that two panoramas of the surface of Venus have shown scenes not unlike those of terrestrial desert landscapes, the next step will be to discover the processes which moulded the landforms of Venus. □

So Madagascar was to the north

from Peter J. Smith

IN a recent article on the Madagascar controversy, Kent and Tarling (*Nature*, 261, 304; 1976) were adamant in claiming that "the Madagascar controversy still lives". They were right of course, even excluding the element of self-fulfilling prophesy. To expect everyone to accept that "palaeomagnetism has provided the definitive answer" to the Madagascar problem (*Nature*, 259, 80; 1976) was evidently overoptimistic, or at least premature. The controversy does indeed live. But for how much longer in the light of a new palaeomagnetic analysis from McElhinney *et al.* (*Geology*, 4, 455; 1976)?

The long-standing argument is, of course, over the geographical position of Madagascar before the breakup of Gondwanaland. Did this large island lie in the coastal embayment of Mozambique some 4° south of its present position? Or was it about 15° north, adjacent to the coast of Kenya-Tanzania-Somalia? Or has it always been where it is with respect to Africa, except perhaps for just a small amount of eastward drift? The answer is critical in defining the fit of eastern and western Gondwanaland. Evidence of various types has been adduced in favour of all three positions at various times, although clearly no more than one of the options can be correct.

The problem seemed well on the way to solution earlier this year when Embleton and McElhinney (*Earth planet. Sci. Lett.*, 27, 329; 1976) presented palaeomagnetic data supporting the southward drift of Madagascar—



Fig. 1 Part of Venera 9 panorama. The rotation axis of the telecamera was inclined at ~40° to the vertical axis of the landing stage. The rocks shown here lie on, and form, a steep

slope (the on-board inclinometer reading was ~30°). Data auxiliary to the picture data were transmitted in vertical bands which have been removed.

that is, a predrift position to the north. But Kent and Tarling were not so sure, citing a major geological argument against a northern position. In the vicinity of Kenya-Tanzania there are thick sediments apparently deposited from the Upper Carboniferous onwards, both onshore and out to several hundred kilometres offshore. So how, Kent and Tarling asked, could these deposits "exist in their present position if this was occupied by a micro-continent that has since moved away"?

They then went on to question both the validity of the palaeomagnetic data and the significance of the analysis based on them. The point about validity hinged on whether or not the measured sediments had been remagnetised since they were laid down. But Kent and Tarling argued that even if remagnetisation could be ruled out and the data were perfectly valid, there is at least one alternative way of comparing the Madagascar data with those from other landmasses. And this other way suggests that a southward drift of Madagascar is no more likely than a northward drift or no drift at all. The point was not that one method is any better or worse than the other, but simply that both are equally valid and reasonable.

Be that as it may, there is now a sequel. Since Embleton and McElhinny reported their first data, palaeomagnetic investigations of Madagascan rocks have continued (both in Australia and France) to the point where there is now an almost complete palaeomagnetic coverage of the Karroo Supergroup from the Upper Carboniferous to the Middle Jurassic. And all of the results have been brought together and analysed by McElhinny *et al.*

The Madagascan data fall into three sets covering, respectively, the time-spans: (1) Upper Carboniferous to Lower Permian, (2) Upper Permian to Lower Triassic and (3) Middle Triassic to Middle Jurassic. Unfortunately, appropriate African data are heavily biased towards the Middle Triassic-Middle Jurassic, which makes comparison over the total Karroo period difficult.

And the results of the comparison are striking. Taking the time-spans (1), (2) and (3) together, the average angular difference between the Madagascan and African-South American pole positions is 16.8° for Madagascar in the southern predrift position and 15.3° assuming the island to have maintained its present position. These are palaeomagnetically significant differences. By contrast, the average angular difference for Madagascar in the northern predrift position is only 4.5°, which is palaeomagnetically insignificant. Moreover, these averages conceal

no anomalies; time-spans (1), (2) and (3) taken separately each give a similar result.

In short, the palaeomagnetic case for the northern predrift position of Madagascar is, in the word of McElhinny and his colleagues, "unequivocal". Even the sceptics must surely agree that at the very least the case is strong. It is difficult to believe that the palaeomagnetic data are not valid. Remagnetisation, for example, is possible, but hardly likely in view of the similar results for the three time-spans and the number of different rock types involved. So it would appear that someone has a lot of reconciling to do. □

Competition and sexual dimorphism in plants

from Peter D. Moore

As members of a species in which males and females are increasingly coming into competition with one another, it is interesting for us to consider the evolutionary consequences which have resulted from such a state of affairs in other species. Sexual dimorphism is the term used for situations in which the male and female of a species are adapted so that they draw on different resources, or tap a resource in different ways. It results from conditions where the limitations of the environment can produce deleterious competitive interactions between males and females inhabiting the same area.

Sexual dimorphism is quite common among animals and may result in considerable differences between the sexes. In many birds of prey, such as the sparrow hawk (*Accipiter nisus*) the male is considerably smaller than the female. The two sexes feed on different prey species, the male concentrating on smaller, more agile species, and the female on larger ones.

Among plants, very few examples of sexual dimorphism have been described. Obviously it could exist only within dioecious species, where the sexes are separate. One example is the sheep's sorrel (*Rumex acetosella*) which was studied by Putwain and Harper (*J. Ecol.*, **60**, 113; 1972). Here the two sexes were found growing together, but showed considerable differences in their times of growth and in the allocation of food reserves. Male plants grew faster in the spring, but expended less energy on the production of inflorescences and pollen than did the female on seed production. Female plants were later in their growth, but finally developed a taller canopy. Males

invested more energy in the production of roots and vegetative offshoots. Males and females thus tap the environmental resources at different times and in rather different ways, constituting an example of niche diversification between the sexes.

Freeman, Klikoff and Harper (*Science*, **193**, 597; 1976) have examined several dioecious plant species in relation to soil moisture in arid to semiarid habitats in Utah. They found that the ratio of male:female plants in all species examined varied with environmental conditions, mainly water availability. For example the meadow rue (*Thalictrum fendleri*) had a male:female ratio of almost 7:1 in dry sunny positions, whereas in moist shady conditions it was nearer 1:4. Mormon tea (*Ephedra viridis*) had a male:female ratio of 1.6:1 on steep, arid slopes and about 1:2 on alluvial bottomlands. The salt grass (*Distichlis spicata*) gave a ratio of about 3:1 in very saline conditions and about 1:1.5 in moderately saline areas.

These data suggest that males of all these species survive better than females in conditions of water stress, whereas the reverse is true where water is more available. Alternatively, sex determination may be environmentally controlled. Whatever the cause of this state of affairs, Freeman *et al.* suggest that the differentiation of niches between the sexes has adaptive significance. Reproductive success in males in these wind-pollinated species may be enhanced by their being located on exposed, dry ridges. Seed output from females, on the other hand, would be greater in conditions of improved water supply, especially later in the season.

They have not examined the role of vegetative reproduction in determining sex ratios. Putwain and Harper showed the importance of this influence in their *Rumex* studies and it is possible that resource allocation to vegetative reproduction in these species varies between the sexes and is differentially affected by water availability. *Ephedra*, for example, spreads extensively by vegetative reproduction. Niche differentiation between the sexes of these plants has evidently occurred in response to a selective pressure, but the precise physiological or morphological cause of the variations in sex ratio remain unclear.

Dioecious plant species are not uncommon and it will be interesting to see whether more of them exhibit sexual dimorphism. Sex ratios in populations, however, may not always be the best guide to dimorphism in plants because of the influence that vegetative reproduction can have by redressing the effects of differential germination and survival. □

review article

Transposable genetic elements and plasmid evolution

Stanley N. Cohen*

Transposable elements of DNA that are structurally defined and genetically discrete units seem to have an important role in the evolution of bacterial plasmids. Recombination occurring at the termini of such elements can result in the joining together of unrelated DNA segments that lack extensive nucleotide sequence homology. In addition, transposable elements serve as novel biological switches capable of turning on and off the expression of nearby genes as a consequence of their insertion into or excision from plasmid genomes.

ALTHOUGH the existence of plasmids in bacteria has been known for more than two decades, the extraordinary prevalence and biological diversity of these extrachromosomal genetic elements has been appreciated only recently. Plasmids were first identified in the Enterobacteriaceae, but subsequently have been found in almost every bacterial group where they have been searched for. Naturally-occurring plasmids vary in size from the 2,250-nucleotide-long minicircular "cryptic" plasmid of *Escherichia coli* strain 15 (ref. 1) to the large and complex F⁺ plasmids², which may contain more than 400,000 nucleotide pairs and carry up to 600 genes. A wide variety of biological functions is specified by plasmids (Table 1).

The essential feature common to all plasmids is a replication region capable of propagating itself and any genes linked to it. Presumably, early steps in plasmid evolution involve the assembly of deoxyribonucleotides to form a site for initiation of DNA replication (the replication origin) and acquisition of genes that specify functions required for autonomous replication. Subsequent linkage of the resulting replication segment to one or more genes that provide a biological advantage to cells carrying the plasmid would be expected to aid its propagation. Later steps in the evolution of some plasmids might involve the addition of groups of genes that facilitate inter-bacterial transfer or an extracellular existence. Thus, in this evolutionary continuum, plasmids can be viewed as primitive bacteriophages that have not yet acquired those specialised functions necessary for a complex replicative cycle or the production of infectious particles capable of existing outside the host cell. Conversely, bacteriophages can be viewed as plasmids in which a basic replication region has become linked to gene segments that specify the additional biological functions needed for the production of phage particles.

Until recently, it has been generally assumed that the structural evolution of extrachromosomal elements has occurred by mechanisms of "general" or "ordinary" recombination. In *E. coli*, general recombination involves the bacterial *recA* gene product, and is commonly dependent on physical breakage and reciprocal exchange of DNA sequences in a region of extensive genetic homology³. While

this mechanism has undoubtedly played some part in plasmid evolution, experiments carried out during the past several years have shown that the acquisition of certain new segments of genetic material by plasmid and bacteriophage genomes can occur by "illegitimate"^{4,5} recombination of DNA sequences that have little or no ancestral relationship^{6,7}. Thus, the remarkable structural and genetic diversity of plasmids seems in part to be the consequence of quantal, rather than reciprocal, recombination that involves the joining of termini of separately evolving and structurally defined segments of DNA and the translocation (transposition) of genetic segments from one DNA site to another. Moreover, at least some of the transposable elements involved in the structural evolution of plasmids and other prokaryotic chromosomes can serve also as biological switches capable of turning groups of genes on and off as a consequence of their insertion and excision. Thus, transposable elements seem to have an important role in both the structural organisation and the functional expression of genetic information on DNA.

Segmental composition of large plasmids

Investigations from several laboratories⁸⁻¹² have shown that certain large bacterial plasmids can undergo reversible dissociation into separate units in some bacterial species. For example, "cointegrate"¹³ antibiotic resistance plasmids can dissociate in *Proteus mirabilis* into separate segments that carry genes for transfer functions (the RTF replicon) or for resistance to antimicrobial agents (the R determinant segment)⁸⁻¹². Each of the two component regions of cointegrate plasmids contains its own replication origin¹⁴. Since independently formed isolates of an RTF unit derived from the same plasmid seem to be genetically and structurally identical^{15,16}, dissociation and reassociation (that is, recombination) of cointegrate plasmids must necessarily involve specific DNA loci. Electron microscope heteroduplex studies of the isolated RTF segment of one antibiotic resistance plasmid has shown that it has considerable DNA nucleotide sequence homology with the RTF regions of several other cointegrate plasmids¹⁷. Moreover, the RTF component of the antibiotic resistance plasmid was found to share much homology with the corresponding segments of the F and ColV-K94 plasmids.

Further heteroduplex mapping of several phenotypically

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Table 1 Some properties coded by naturally occurring plasmids

Property	Exemplified by
Fertility—ability to transfer genetic material by conjugation	F, R1, Col1
Production of bacteriocins	CloDF13 (<i>Enterobacterium cloacae</i>) ColE1
Antibiotic production	SCP1 plasmid of <i>Streptomyces coelicolor</i>
Heavy metal resistance (Cd ²⁺ , Hg ²⁺)	pI258 (<i>S. aureus</i>), R6
Ultraviolet resistance	Col1b, R46
Enterotoxin	Ent
Virulence factors, haemolysin K88 antigen	ColV, Hly
Metabolism of camphor, octane, and so on	Cam, Oct (<i>Pseudomonas</i>)
Tumorigenicity in plants	TI-plasmid of <i>Agrobacterium tumefaciens</i>
Restriction/modification	Production of <i>EcoRI</i> endonuclease and methylase by plasmid of RY13

Plasmids listed are indigenous to *E. coli* unless otherwise indicated.

distinct plasmids has indicated that their regions of homology begin and terminate at very precise boundaries¹⁷: segments to one side of the boundary show major differences in DNA nucleotide sequences, whereas segments to the other side are preserved largely intact. R-determinant segments of certain antibiotic resistance plasmids isolated in widely spaced parts of the world were found to be preserved almost intact, but no detectable homology was seen with the corresponding segments of the F and ColV-K94 plasmids. Thus, the different phenotypic properties of these large plasmids seem to result in part from the linkage

of separately derived supplemental gene segments to the basic RTF unit. These observations suggest that different combinations of separately evolving DNA segments that have little or no ancestral relationship may be involved in plasmid formation.

Inverted repeats on plasmid DNA

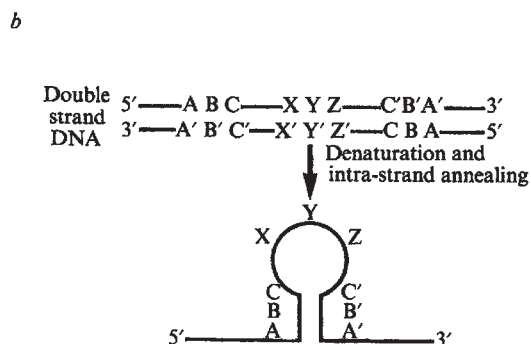
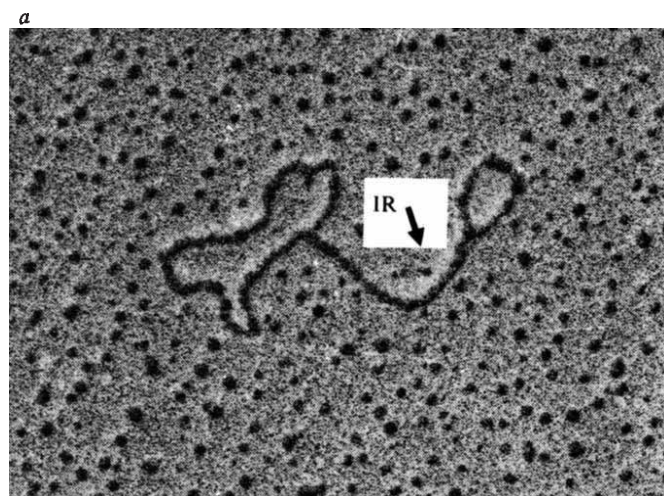
Heteroduplex studies of plasmid DNA sequence relationships by Sharp *et al.*^{17,18} also resulted in the discovery of inverted repeats on plasmid DNA. Inverted repeats consist of DNA segments that are duplicated in reverse nucleotide sequence orientation along the genome (Fig. 1). Because of the antiparallel nature of DNA polynucleotide chains, the nucleotide sequences contained within inverted repeats are identical on each of the DNA strands from the 3' to 5' directions; consequently, a structurally defined DNA sequence and its complement exist on each strand of the DNA molecule. During heteroduplex analysis, intra-strand annealing of the primary and repeated complementary sequences results in the formation of hairpin-loop or foldback structures. The complementary sequences are contained in the stalk of such structures, whereas intervening non-duplicated sequences form the loop part. An axis of twofold rotational symmetry bisects the palindromic stem that is formed by intrastrand annealing of the inverted repeat sequences during formamide spreading of DNA for heteroduplex analysis. In at least two instances, antibiotic resistance genes carried by plasmids (that is, the genes specifying resistance to tetracycline and kanamycin-neomycin) were shown to map in the immediate vicinity of inverted repeat structures¹⁷.

Insertion sequence (IS) regions on plasmids

One of the hairpin-loop structures located on the R6 and R6-5 plasmids was found to have another structural feature of special interest. R6-5 is a spontaneously isolated variant of R6; although it does not express the tetracycline (Tc) resistance of the parent plasmid, R6-5 was nevertheless found to contain the hairpin-loop structure associated with the Tc-resistance gene of R6¹⁷. Electron microscope heteroduplex analysis of the two plasmids indicated that the Tc-sensitive R6-5 plasmid contains all of the DNA sequences present in the Tc-resistant R6 plasmid—plus an additional segment of DNA 1.4 kb in length¹⁷. Spontaneous excision of the segment from the R6-5 plasmid, which occurs at low frequencies during normal growth of bacteria carrying the plasmid and at higher frequency¹⁹ after transformation²⁰ of *E. coli* by R6-5 plasmid DNA, leads to regeneration of a plasmid that is phenotypically and structurally identical to the tetracycline-resistant R6 parent. These results indicate that a Tc-resistance gene is present on the R6-5 plasmid, but that it is being inactivated reversibly by insertion of an additional segment of DNA¹⁷.

The inserted DNA segment that turns on and off the expression of tetracycline resistance in the R6-5 plasmid was shown to be a direct duplication of a structurally defined nucleotide sequence that is repeated again in

Fig. 1 Inverted repeats. *a*, Electron photomicrograph of formamide-spread²¹ "mini-plasmid" derivative of the pSC105 plasmid²⁰. The inverted-repeat units forming the stalk (IR) of the Km-resistance hairpin-loop structure are indicated. *b*, Schematic representation of formation of hairpin-loop structure showing palindromic stem. The DNA nucleotide sequences *ABC* are repeated in inverted orientation on this DNA. Following DNA denaturation and strand separation, hairpin-loop structures can be formed by intrastrand annealing of the complementary nucleotides. The sequence bracketing the base of the stem forms an axis of twofold rotational symmetry.



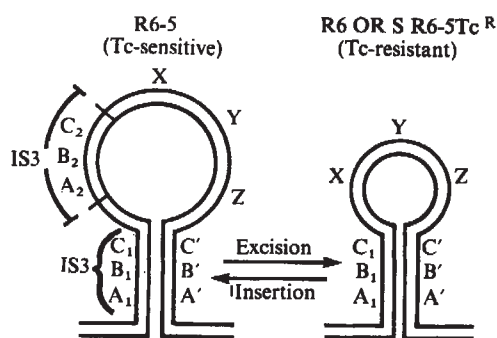


Fig. 2 Turn on and turn off of Tc-resistance gene of R6/R6-5 plasmid by excision and insertion of repeated DNA sequence. The nucleotide sequences ABC and $C'B'A'$ form a hairpin-loop structure by intrastrand annealing during spreading of plasmid DNA in the presence of formamide. Insertion of the sequence $A_2B_2C_2$, which is a direct repeat of $A_1B_1C_1$ results in loss of expression of the nearby Tc-resistance gene. Excision of this sequence leads to expression of Tc resistance by the plasmid.

reverse orientation further along the plasmid genome to form the stalk of the hairpin-loop structure near the Tc-resistance gene (Fig. 2). Insertion and excision of this segment of DNA at a site within the loop affects expression of the nearby Tc-resistance gene.

Inactivation of genes as a consequence of insertion of DNA sequences of defined length has been recognised for about a decade. Insertion sequence (IS) elements (for review, see ref. 7), which range in length from 800 to 1,400 base pairs, have been known to become integrated at multiple sites in bacterial and phage genomes and to produce polar mutations²¹⁻²³; insertion of an IS element not only abolishes the function of the gene into which the element is inserted, but it can also affect functioning of genes distal to the insertion site with respect to the promoter of the operon. Moreover, at least one IS element carries a promoter region, and is capable of turning on or off the activity of adjacent genes according to the direction of insertion of the IS segment²³. The 1.4-kb DNA segment observed to affect expression of tetracycline resistance in the R6/R6-5 plasmid as a consequence of its insertion or

excision seemed to be similar in its characteristics to the IS elements¹⁷.

Subsequent electron microscope heteroduplex studies²⁶ have shown that the DNA sequence that forms the stalk of the Tc-resistance hairpin-loop structure of the R6 plasmid, and which is also the inserted DNA segment affecting expression of Tc resistance, is one of the previously identified IS regions—IS3. These studies have involved the use of previously characterised^{27,28} λ phage mutants carrying the known IS regions; separated strands of phage λ DNA containing IS1, IS2 or IS3 were annealed with R6-5 plasmid DNA, and homology of the IS3 element with the sequence of the Tc-resistance stalk was shown²⁶.

Parallel investigations by Hu *et al.*¹⁸ have shown that IS1 and IS2 elements are also present on R6 and R6-5 plasmids, and on the related plasmid R100. IS2 exists as a simple insertion within the RTF unit. Two copies of IS1 exist as direct repeats at the boundaries of the RTF and R-determinants of these plasmids, and by analogy from the data by Sharp *et al.*¹⁷, exist also at corresponding locations of certain other cointegrate antibiotic resistance plasmids that are homologous with R6 in these regions. Using similar methods, Ptashne and Cohen²⁶ also demonstrated the presence of directly repeated IS1 insertions at the junction of the RTF and R-determinant units of R6-5.

The discovery of directly repeated copies of the IS1 element at the two junctions of the RTF and R-determinant components of cointegrate antibiotic resistance plasmids provides a possible mechanism for the previously reported reversible dissociation of such plasmids into separate segments⁸⁻¹¹. The IS1 elements also provide a possible mechanism for amplification of the R-determinant segment and the formation of plasmids consisting of polygenic R-determinant units. As proposed by Hu *et al.*¹⁸ and by Ptashne and Cohen²⁶, multiple IS1 copies of and R-determinant units could accumulate on cointegrate R-plasmids by recombination involving the IS1 element. According to this model, the IS1 elements could also mediate dissociation of the component of the plasmid: the number of R-determinant copies included on each of the component replicons would depend on which IS1 regions participate in the recombinational event. Recombination between the most distant pair of R-determinant units as

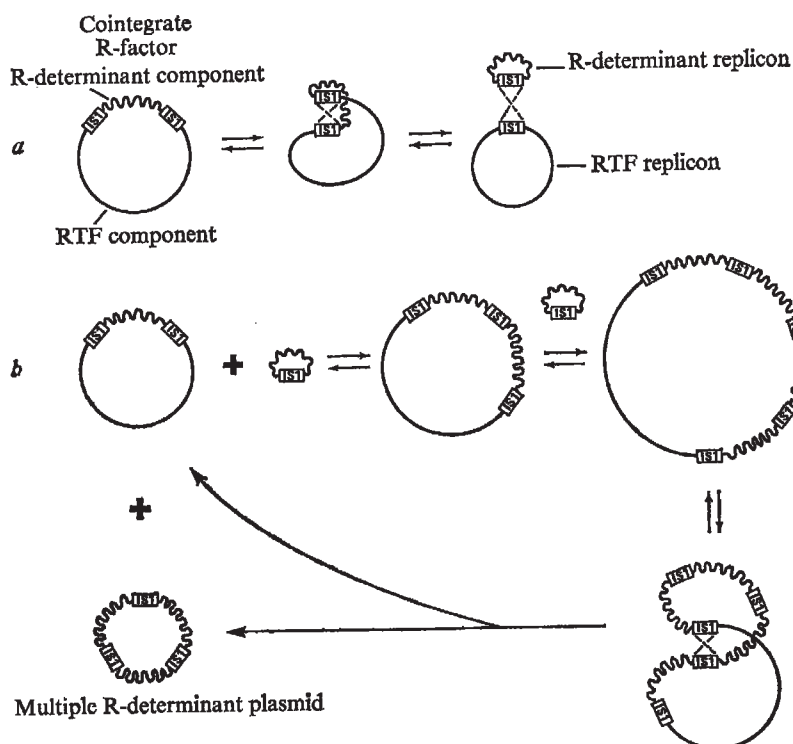


Fig. 3 Proposed mechanism^{26,29} for reversible dissociation of cointegrate R plasmids at sites of IS1 elements. From Ptashne and Cohen (ref. 26). The model depicted provides for the formation of independent RTF and R-determinant replicons (a) and for the production of polygenic R-determinant units. (b). The model is schematic; it has not been determined whether both the RTF and R-determinant units receive IS elements during dissociation of cointegrate R plasmids or whether the two IS1 elements are distributed asymmetrically.

shown in Fig. 3 would yield an RTF replicon plus a poly-R-determinant plasmid, whereas recombination between intermediately located IS units would yield a DNA molecule containing both an RTF segment and a varying number of R-determinant copies²⁶. Thus, separate RTF and R-determinant replicons⁸⁻¹¹, as well as plasmids containing multiple R-determinant copies³⁰ could be produced from cointegrate R plasmids by the same recombinational process.

IS elements also seem to be involved in the recombination of F plasmids with chromosomal genes to yield F' plasmids³¹⁻³³. The electron microscope heteroduplex studies of Hu and Davidson and their associates³¹⁻³³ have identified three structurally defined DNA segments of F that are hotspots for illegitimate recombination events that do not require bacterial *recA* gene function. One of these, the $\epsilon\zeta$ sequence, is homologous with IS2 (ref. 29). A second recombinationally active segment on F, the $\alpha\beta$ sequence, is indistinguishable from the IS3 element³³. Formation of an Hfr cell by integration of F into the chromosome can occur by interaction of the plasmid with an IS element inserted in either of two polarities. In 23 of 25 independently derived Hfrs, the point of origin and the direction of transfer of the chromosome by the integrated F plasmid could be predicted by the polarity of the $\alpha\beta$ or $\epsilon\zeta$ sequence on the

plasmid^{32,33}. Taken together, these various data strongly suggest that IS sequences are involved in Hfr formation. These studies³¹⁻³³ suggest also that IS elements have a prominent role in the acquisition of chromosomal genes by plasmids and indicate that deletions of F-plasmid DNA can occur during F'-plasmid formation, and that one endpoint of the deleted DNA segment often is coincident with the endpoint of an IS element.

Translocation (transposition) of DNA segments carrying antibiotic resistance genes

In 1964 Dubnau and Stocker³⁴ reported that antibiotic resistance genes from a plasmid could become associated with the P22 prophage and subsequently attach to the chromosome of *Salmonella typhimurium*. Kondo and Mitsuhashi observed that *E. coli* phage P1 could similarly receive a resistance gene from a plasmid³⁵. Later, Mitsuhashi and coworkers^{37,38} described the spontaneous integration of R-plasmid genes for chloramphenicol (Cm) resistance at various sites of the *E. coli* chromosome, and the subsequent movement of the Cm resistance trait on to another R plasmid or on to bacteriophage P1, which could then transfer the resistance interbacterially. Other observations from the same laboratory suggested that Tc-resistance genes

Abbreviated Rules of Nomenclature for transposable elements of DNA*

Definition

Transposable elements are DNA segments which can insert into several sites in a genome.

Classes of element

(1) IS (simple insertion sequences): contain no known genes unrelated to insertion function, generally shorter than 2 kb. Symbols: IS1, IS2, IS3 and so on.

(2) Tn (more complex, transposable elements, often containing IS elements): behave formally like IS elements, but contain additional genes unrelated to insertion function, generally larger than 2 kb. Symbols: Tn1, Tn2, Tn3 and so on. A different number is assigned to each independent isolate from nature, even if it is apparently identical to some previous isolate. The designations assigned in the accompanying table to some known Tn elements should be used in all publications.

(3) Episomes (complex, self-replicating elements often containing IS and Tn elements).

Organisms and genomes with inserted elements

(1) Organisms. When an inserting element has been introduced into a previously described bacterial strain, the new strain should be given an isolation number. Its genotype can be denoted by the genotype of the parent strain, followed by the name of the element in parentheses.

(2) Genomes. If location is known, specify gene or region in which element is inserted, followed by a number designating the particular insertion mutation, then by double colon and finally by name of inserted element; for example, *galT-236::IS1* (IS1 within gene *galT*).

Central registry

To avoid duplications of numbers, all new IS and Tn

elements should be checked with a central registry before numerals are assigned to them in publications. A registry for Tn elements will be maintained by Esther Lederberg, Department of Medical Microbiology, Stanford University Medical School, Stanford, California 94305 (USA) as part of the Plasmid Reference Center.

Designation of Tn elements*

Element†	Plasmid origin‡	Resistance markers§	References
Tn1	RP4	Ap	6
Tn2	RSF1030	Ap	7
Tn3	R1	Ap	8
Tn4	R1	ApSmSu	8
Tn5	JR67	Km	2
Tn6	JR72	Km	2
Tn7	R483	TpSm	1
Tn9	pSM14	Cm	4
Tn10	R100	Tc	3, 5

†The symbol Tn8 has not yet been assigned.

‡Natural plasmid from which element originated.

§Ap = ampicillin; Sm = streptomycin; Su = sulphonamide; Km = kanamycin; Tp = trimethoprim; Tc = tetracycline.

¹Barth, P. T., Datta, N., Hedges, R. W., and Grintner, N. J., *J. Bact.*, **125**, 800-810 (1976).

²Berg, D. E., Davies, J., Allet, B., and Rochemaux, J., *Proc. natn. Acad. Sci. U.S.A.*, **72**, 3628-3632 (1975).

³Foster, T. J., Howe, T. G. B., and Richmond, K. M. V., *J. Bact.*, **124**, 1153-1158 (1975).

⁴Gottesman, M., and Rosner, J. L., *Proc. natn. Acad. Sci. U.S.A.*, **72**, 5041-5045 (1975).

⁵Kleckner, N., Chan, R., Tye, B., and Botstein, D., *J. molec. Biol.*, **97**, 561-575 (1975).

⁶Hedges, R. W., and Jacob, A. E., *Molec. gen. Genet.*, **132**, 31-40 (1974).

⁷Heffron, F., Sublett, R., Hedges, R. W., Jacob, A., and Falkow, S., *J. Bact.*, **122**, 250-276 (1975).

⁸Kopecko, D. J., and Cohen, S. N., *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1373-1377 (1975).

*Prepared by a committee consisting of A. Campbell (chairman), D. Berg, D. Botstein, R. Novick and P. Starlinger. The complete report will appear in *DNA Insertion Elements, Plasmids and Episomes* (Cold Spring Harbor Laboratories, Cold Spring Harbor, in the press).

were also capable of moving from plasmid to plasmid or from plasmid to chromosome³⁹⁻⁴¹.

In 1968, Anderson *et al.*⁴² reported observations suggesting that an Ap-resistance determinant from one plasmid could become associated with another plasmid. Datta *et al.*⁴³ observed an apparently similar recombinational event that resulted in acquisition by the R64 plasmid of the TEM β -lactamase (Ap resistance) gene originally present on another plasmid, RP4; Richmond and Sykes⁴⁴ subsequently reported that the TEM β -lactamase gene derived from the related RP1 plasmid could integrate into the *E. coli* chromosome. Hedges and Jacob⁴⁵ isolated a series of plasmid derivatives that had received the Ap-resistance trait from RP4, and found that an increase in plasmid molecular weight accompanied the change to Ap resistance; other plasmids were then able to acquire the TEM β -lactamase resistance from the derivative plasmid. These authors concluded that the structural information for the TEM β -lactamase was carried by a transposable DNA sequence that they termed transposon A (TnA)⁴⁶; Heffron *et al.*⁴⁶ subsequently demonstrated that the nucleotide sequences of the TnA segment, which is approximately 3×10^6 daltons in size, are common to naturally occurring plasmids of a variety of compatibility groups that specify the TEM type Ap resistance.

Although these recombinational events apparently involved movement of genetic information from one genome to another, they seemed to involve *recA*-dependent general recombination. Other experimental evidence, however, has demonstrated that translocation of structurally defined segments of DNA carrying antibiotic resistant genes can occur by terminus-site-specific *recA*-independent recombination. Some of these events involve known IS elements, which can also move from genome to genome independently of *recA* function⁴⁷; others seem to be associated with newly observed structurally defined segments of DNA that have been found on plasmids as inverted repeats.

During studies of mobilisation of a non-conjugative plasmid by a conjugally proficient Ap-resistance plasmid¹⁹, a recombinant DNA molecule was isolated, which on further study was shown by electron microscope heteroduplex analysis to have been formed by translocation of a specific DNA segment from one plasmid to another¹⁹. Translocation of this DNA segment between plasmids did not require the bacterial *recA* gene product, suggesting that it did not involve ordinary recombination mechanisms. The event was site specific so far as the transposable element itself is concerned (that is, the element could move between plasmids as a discrete structural unit), but the element could become inserted at different sites on the recipient pSC101 plasmid. Heteroduplex analysis showed that all of the sequences present in the translocating Ap-resistance segment were derived from a continuous region of the donor plasmid, and that insertion of the element into the recipient plasmid could occur in either direction¹⁹. Moreover, a 130-nucleotide long inverted repeat DNA sequence was present at the termini of the 4.5-kb transposable unit.

A schematic representation of these events is shown in Fig. 4. In effect, the plasmid DNA originally located at the ends of the inverted repeat termini of the transposable element is replaced by the recipient plasmid during the translocation event; it is not known, however, whether the transposable unit exists in a physically separate state during the process. In the hairpin-loop structure configuration, (Fig. 4a) the termini of the inverted repeats forming the stem of the Ap-resistance transposable segment are located around an axis of twofold rotational symmetry that marks the site of excision of the element from one plasmid and insertion of the element into another. Alternatively, such molecules may form loop configurations (Fig. 4b) similar to that proposed by Campbell⁴ for integration of phage λ into the *E. coli* chromosome, or may form cruciform structures³⁰. Although

the actual structural configuration for the transposable segment during the recombinational event is not known, it seems plausible that enzymes capable of recognising the inverted repeat termini of the element may be involved in translocation.

Subsequent experiments⁵¹ have indicated that the structurally defined Ap-resistance element observed to translocate from the pSC50 plasmid on to plasmid pSC101⁴⁹ is indistinguishable by electron microscope heteroduplex analysis from the TnA element from R1-19 studied by others^{46,52}. Heffron and coworkers⁵² have shown that insertion of TnA into a plasmid results in polar mutations similar to those occurring as a consequence of insertion of IS units. Using heteroduplex mapping, these investigators⁵² have also demonstrated that the TnA element can insert into multiple sites clustered in a region corresponding to only one-third of the length of the 5.5×10^6 dalton RSF1010 plasmid genome. Similar clustering of sites that have received TnA has been observed on the ColE1 plasmid⁵³ and on pSC101⁵¹.

Other recent genetic and molecular studies have shown that TnA is one of a number of antibiotic resistance-gene segments of DNA that can undergo *recA*-independent translocation. In 1972, Chan *et al.*⁵⁴ observed that growth of the *S. typhimurium* phage P22 in a bacterial strain carrying the R100 plasmid resulted in formation of an unusual phage variant that could transduce resistance to Tc at a high frequency. The Tc-resistant P22 phage seemed analogous to the antibiotic-resistant P22 phage observed by Dubnau and Stocker⁵⁴; moreover, it did not seem to result from the mechanism normally responsible for the formation of P22 transducing phage, since insertion of Tc resistance into the phage genome did not occur at the site usually associated with attachment of the phage to the *Salmonella* chromosome.

The Tc-resistance DNA segment that had recombined with phage P22 was found to contain a hairpin-loop structure⁵⁵ similar to the one identified previously on the R6-5 plasmid¹⁷. Subsequent studies⁵⁶ have demonstrated that this structure, which has inverted repeats of the IS3 element at its termini⁵⁶, is capable of translocation from P22 on to many different sites of the *Salmonella* chromosome. Insertion of the transposable element into a structural gene abolishes the function of that gene; moreover, a polar effect on the expression of genes distal to the promoter occurs when the element is inserted into a group of genes that form a single transcription unit⁵⁶, as is characteristic of the IS elements⁷. The two inverted repeat copies of IS3 near the Tc-resistance gene seem to be able to undergo translocation concurrently and to carry along the intervening segment of DNA.

Insertion of the Tc-resistance hairpin-loop structure occurs without loss of genetic information from the molecule into which it inserts; most of the mutated bacteria formed as a consequence of insertion can revert to prototrophy⁵⁶. However, excision of the transposable element is usually not precise. Although insertion of the Tc-resistance element into the *Salmonella* chromosome results in a distribution of auxotrophic mutants similar to that obtained by chemical mutagenesis⁵⁶, clustering of insertion sites within the histidine operon suggests that insertion may not be entirely random. As in the case of the TnA element, insertion and excision of the Tc-resistance (IS3) segment is independent of the function of the *recA* gene of the host^{56,57}.

Berg *et al.*⁵⁸ have made analogous observations involving a segment of plasmid DNA that carries genes for resistance to the antibiotic kanamycin (Km). This segment contains an inverted repeat hairpin-loop structure similar in appearance to, but different in size from, the structure previously associated with Km resistance on various R plas-

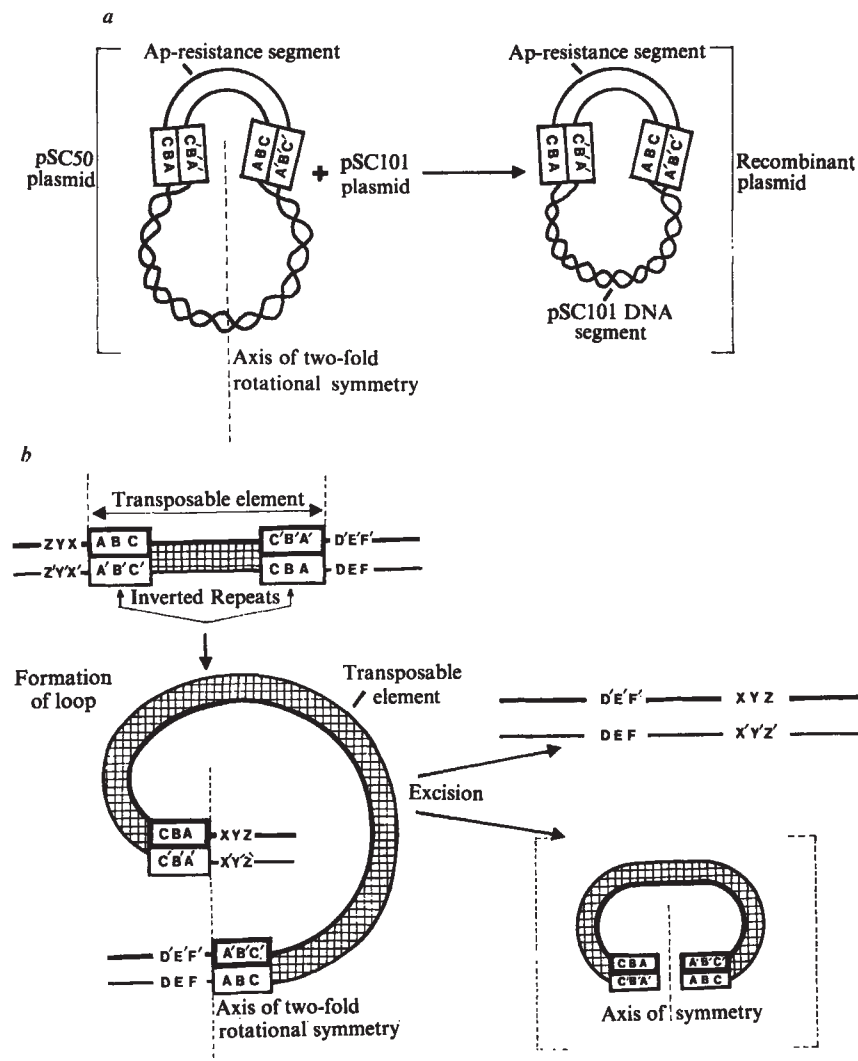


Fig. 4 Translocation of Ap-resistance DNA segment (TnA) from the pSC50 plasmid to pSC101. *a*, Schematic representation showing the Ap-resistance hairpin-loop structure with a 130–140 nucleotide-long inverted repeat stem. As a consequence of translocation, the pSC50 plasmid DNA at the base of the stem is replaced by the pSC101 plasmid (that is, the Ap-resistance segment is translocated from pSC50 to pSC101). Translocation of the hairpin-loop structure involves the ends of the palindromic (stem) sequences that bracket an axis of twofold rotational symmetry. The molecular nature of the DNA product resulting from excision of the Ap-resistance segment from pSC50 is not known. *b*, Possible Campbell model configuration for DNA segment involved in translocation. There is currently no evidence that the same event results in both translocation and restoration of DNA nucleotide sequence continuity at the site into which the Ap-resistance segment has been inserted; although excision with reversion to prototrophy, and translocation are both observed, these outcomes may represent separate events that occur on different DNA molecules, as appears to be the case with bacteriophage μ ^{82,83}. The separated Ap-resistance transposable DNA segment is shown in brackets, since its existence as a separate unit has not been demonstrated.

mids¹⁷. The segment behaves functionally like the Tc-resistance and TnA elements; it can insert at several different sites on phage λ and excision leads to recovery of an intact phage genome. A second Km-resistance DNA segment lacking inverted repeat termini was also observed¹⁸ to be capable of translocation from R plasmids to bacteriophage λ , suggesting that inverted repeats may not be essential for the translocation event or that one of the limbs of the inverted repeat is lost during translocation of this particular element.

Another translocating segment of DNA carrying an antibiotic resistance gene but lacking inverted repeats at its ends has been reported by Gottesman and Rosner¹⁹. This element had originally been acquired by *E. coli* bacteriophage P1 from the antibiotic resistance plasmid R10 (ref. 35). It was subsequently transferred to phage λ by a process shown to be independent of host *rec* or λ *red* recombination functions; however, possible involvement of P1 recombination functions in the translocation event was not excluded. Very recent investigations (MacHattie and Jackowski, personal communication) indicate that this Cm-resistance translocating segment of DNA contains directly repeated copies of the IS1 element at its termini.

During the studies of Kopecko and Cohen²⁰, a second transposable element that carries resistance to sulphonamide (Su), streptomycin (Sm) and Ap was observed to move from the pSC50 plasmid to pSC101. Subsequent experiments have shown that this 20.4-kb unit, which has been named TnS, contains the TnA element within it²¹. Inverted repeat segments, 130–140 nucleotides in length, are present at the termini of TnS as well as at the ends of TnA. More-

over, the termini of the TnS element seem to be highly interactive "hotspots" for additional illegitimate recombinational events involving the deletion or insertion of other segments of plasmid DNA²¹. Recently, a segment of DNA carrying genes for resistance to trimethoprim, streptomycin and spectinomycin has also been identified (TnC, ref. 60). Although it is not yet known whether this unit contains either a direct DNA repeat of inverted repeats at its terminal, it seems to be functionally similar to the other transposable antibiotic resistance units described above.

At this time, structurally defined DNA segments coding for resistance to ampicillin, streptomycin-spectinomycin, sulphonamide, chloramphenicol, tetracycline, and trimethoprim have been shown to be capable of translocation from genome to genome, and it seems that the sequential translocation of such segments may be the principal mechanism by which plasmids can accumulate multiple antibiotic resistance determinants. It seems likely that the widespread clinical and veterinary use of antimicrobial drugs has resulted in the biological selection of plasmids that acquire such transposable elements. Possibly, translocation of additional segments of DNA carrying various other plasmid or chromosomal genes remains undetected because comparable selective pressures have not been applied.

While inverted repeats have not been found on all of the antibiotic-resistance transposable DNA studied, they nevertheless seem to be involved prominently in the translocation process. In bracketing an axis of twofold rotational symmetry at the site of recombination of their termini, inverted repeats form a palindromic DNA sequence as noted above. Palindromes have also been observed at the

DNA interaction site for the *ter* enzyme of phage λ^{a1} , at promoter and operator sites associated with λ CI (ref. 62) and *E. coli lac*⁶³ operon repression, and at the sites of action of several restriction and modification enzymes^{64,65}, suggesting that they may provide highly specific recognition sites for protein-DNA interactions. Observations which show that certain palindrome-site-specific restriction endonucleases are coded by plasmid genes⁶⁴ raise the possibility that functionally analogous plasmid-specified enzymes may have a role in recombinational events involving transposable elements. Moreover, recently obtained data⁶⁶ suggest that genetic information required for translocation of at least one of the antibiotic-resistance transposable elements (that is, TnA) is contained within the loop portion of the element itself. It remains to be determined whether other Tn units carry genes that specify products involved in translocation events.

Promotion of other illegitimate recombination events by transposable elements

Reif and Saedler⁶⁷ have reported that the IS1 element can promote *recA*-independent deletion of DNA sequences immediately adjacent to the IS1 terminus. One endpoint of the deletion seems to be the terminus of IS1 itself, whereas the other endpoint is variable. Recent evidence from other laboratories suggests that transposable segments of DNA carrying antibiotic resistance genes can also promote deletion formation, and that such deletions occur at the termini of the inverted repeats. During heteroduplex analysis of the R100 plasmid, Hu *et al.*⁶⁸ observed that a transfer-deficient R100 derivative had deleted a DNA segment adjacent to the (IS3) terminus of the Tc-resistance hairpin-loop structure. Foster and Willets⁶⁹ subsequently found that following the loss of Tc resistance by R100-1, deletions extending into the adjacent *tra* (transfer) region of the plasmid are observed. Kleckner, Reichardt and Botstein (personal communication) have seen deletion formation also during excision of the Tc-resistance hairpin-loop structure from the *his* region of the *E. coli* chromosome, and D. Berg and B. Allet (personal communication) have observed small deletions in the λ phage chromosome following excision of a Km-resistance transposable element from this DNA segment.

The 130-140-nucleotide long inverted repeats at the termini of TnS also seem to be involved in deletion formation^{71,69}. The pSC50 plasmid, which contains both the TnA and TnS translocating elements, is a Km-sensitive derivative of the Km-resistant R1-19 plasmid⁶⁹. Heteroduplex analysis of pSC50 and its parent indicate that the entire segment of DNA located between a terminus of the TnS unit and a copy of IS1 is lost during the formation of pSC50, bringing the termini of TnS and IS1 in juxtaposition⁷¹. The mechanism by which transposable elements can promote deletion of adjacent DNA sequences is unknown. In at least some instances, however, deletion occurs while the transposable unit is *in situ*, and not at the time of excision or insertion of the element⁶⁹.

Conclusions

As a consequence of work carried out in a number of laboratories during the past several years, an understanding of the organisation and structural evolution of bacterial plasmids has begun to emerge. Terminus-site-specific recombination, which in *E. coli* and *Salmonella* is independent of bacterial *recA* gene function, seems to have an important role; in fact, the addition of structurally-defined DNA segments on to plasmid, phage and bacterial chromosomes, and the excision of specific DNA segments from such chromosomes may represent the principal mechanism by which the organisation of prokaryotic (and perhaps eukaryotic) DNA has evolved. Certainly, this mechanism

seems to account significantly for the structural and genetic diversity of plasmids, and to explain at least some types of interaction between extrachromosomal segments of DNA and the host cell chromosome.

Terminus-site-specific recombination also provides a potential mechanism for the exchange of genetic information between diverse biological species found in nature. There has been recent evidence that an Ap-resistance DNA segment found in *H. influenzae* is structurally and genetically similar to the TnA element isolated from *E. coli* plasmids⁷⁰. In addition, translocation of prokaryotic gene segments of eukaryotic DNA sequences cloned in bacteria has been observed (A. C. Y. Chang and S. N. Cohen, unpublished data); it is tempting to speculate that prokaryotic-eukaryotic genetic recombination involving transposable genetic elements may occur under some circumstances in nature.

In addition to having an important role in the structural organisation of genes on prokaryotic genomes, transposable segments of DNA function as controlling elements or "switches" in turning on and off the expression of plasmid and chromosome genetic information. The remarkable dual role of these elements in affecting both the organisation and function of prokaryotic genomes is perhaps best illustrated by the Tc-resistance transposable segment. Inverted repeats of the IS3 element at the ends of this segment can accomplish movement of the intervening Tc-resistance gene between plasmids, chromosome, and phage, and can also lead to deletion of DNA sequences adjacent to the termini; another repeated copy of IS3 controls expression of the Tc-resistance gene contained within the segment of DNA that is translocated. The demonstration that still other insertion sequence elements carry promoter sites⁷³ for initiation of RNA synthesis provides another potential mechanism by which these units can function as moveable and invertible switches in the evolution of plasmid operons.

Parallels in the effects produced by transposable genetic elements in plasmids, and phenomena observed in eukaryotic systems suggest that similar DNA segments may have a role in the organisation and control of expression of genetic information in higher organisms. The transposable "controlling" elements that regulate phenotypic expression in maize⁷¹⁻⁷⁴ are known to be capable of movement to various sites on maize chromosomes; as a consequence of transposition to different locations, they can exert their controlling influence on a variety of genes. As is the case with the inverted repeats identified on transposable antibiotic resistance segments, transposable maize elements seem to promote deletions of various lengths which affect neighbouring genes. Insertion and excision of repeated DNA sequences has been shown recently also to be associated with genetic instability of the *white* locus on the X chromosome of *Drosophila melanogaster*^{75,76}. The mutations produced in this locus can be transposed to another linkage group, leaving behind a genetic deletion. Moreover, fold-back DNA characteristic of inverted repeats has been found in various eukaryotic chromosomes, and visualisation of this DNA in the electron microscope shows hairpin-loop structures similar to those seen on plasmids⁷⁷⁻⁷⁹.

Analogies between phenomena observed in eukaryotic systems and the transposable genetic elements and inverted repeats identified on plasmids are presently only speculative: nevertheless, bacterial plasmids may prove to be a highly useful model system for elucidating mechanisms involving the organisation and control of genetic information on the complex chromosomes of higher organisms.

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articles

Fission-track dating of pumice from the KBS Tuff, East Rudolf, Kenya

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Fission-track dating of zircon separated from two pumice samples from the KBS Tuff in the Koobi Fora Formation, in Area 131, East Rudolf, Kenya, gives an age of 2.44 ± 0.08 Myr for the eruption of the pumice. This result is compatible with the previously published K-Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum estimate of 2.61 ± 0.26 Myr for the KBS Tuff in Area 105, but differs from the more recently published K-Ar date of 1.82 ± 0.04 Myr for the KBS Tuff in Area 131. This study does not support the suggestion that pumice cobbles of different ages occur in the KBS Tuff.

THE Plio-Pleistocene sedimentary sequence in the East Rudolf basin of Northern Kenya, has been the site of many important hominid¹, archaeological² and palaeontological finds³. Intercalated with the sequence of lacustrine, deltaic and fluvial sediments are a series of prominent felsic volcanic tuffs. These volcanic units have been used to establish a time framework for the rocks of the East Rudolf Basin⁴. K-Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ dating techniques have been applied to >100 rock and mineral samples from East Rudolf, but interpretation of the dates determined by these methods has not been straightforward. Geological^{5,6} and

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analytical^{18,9} factors have been postulated to explain the scatter of K-Ar and ⁴⁰Ar/³⁹Ar apparent ages obtained from volcanic sanidine-anorthoclase crystals separated from pumice cobbles in the tuffs.

The KBS Tuff, lying within the Lower Member of the Koobi Fora formation, has attracted considerable interest amongst palaeontologists and anthropologists because it contains an important stone artefact site² and overlies the bed which contained the hominid cranium KNM ER 1470 (ref. 1). In 1970, Fitch and Miller⁶ published a K-Ar and ⁴⁰Ar/³⁹Ar age spectrum analysis of fresh sanidine crystals separated from pumice cobbles present in the KBS Tuff in Area 105, giving a preliminary date of 2.61 ± 0.26 Myr for the KBS Tuff horizon. Recomputation of this data using improved ⁴⁰Ar/³⁹Ar techniques, together with additional ⁴⁰Ar/³⁹Ar dating studies leads Fitch *et al.*⁷ to the conclusion that a more precise minimum age for the KBS Tuff is 2.42 ± 0.01 Myr.

Curtis has described the original 2.61 ± 0.26 Myr date for the KBS Tuff as being much questioned in private anthropological and palaeontological circles⁸. These doubts led Curtis *et al.* to undertake an independent check, using conventional K-Ar dating techniques on sanidine and glass separated from KBS pumice⁹. The tuff known as KBS in Areas 10 and 105 is distinguished by Curtis *et al.*⁹ from the tuff known as KBS in Area 131 on the basis of K-Ar total fusion dates obtained from these areas: 10 and 105 give a mean date of 1.60 ± 0.05 Myr, 131 giving a mean date of 1.82 ± 0.04 Myr. The 2.61 ± 0.26 -Myr date of Fitch and Miller⁶ is explained by Curtis *et al.*⁹ as possibly resulting from the presence of older pumice blocks in the KBS Tuff. Curtis underlines the desirability of checking radiometric dates by the use of two different methods, and he cites the fission-track dating work on the East Rudolf Tuffs being undertaken at Birkbeck College⁸. Because of the great interest in the KBS Tuff horizon and the apparent conflict between the K-Ar and ⁴⁰Ar/³⁹Ar results, we present here the first dates obtained using the independent technique of fission-track dating on zircon from pumice cobbles collected at one locality in the KBS Tuff. This work represents part of a larger fission-track dating study being made on each of the tuff horizons in the East Rudolf Basin. Full publication of the fission-track results will be made on completion of the project. The theory and techniques of fission-track dating have been comprehensively described¹⁰, as has the specific use of zircon in dating young volcanic ash beds¹¹.

Method

The two pumice samples used in this study, FMA 517 and ER 74/131 came from within 5 m of each other, from the KBS Tuff in Area 131. Sample FMA 517 was collected in 1974 by I. C. Findlater, F. J. Fitch and A. J. Hurford, and was used in a further ⁴⁰Ar/³⁹Ar dating study undertaken at Cambridge⁷. FMA 517 consists of several well rounded

pumice lumps collected within 4 m of each other. The pumice is rich in euhedral feldspar phenocrysts and contains glass showing partial replacement by calcite. Sample ER 74/131 was collected by T. E. Cerling and was used in the K-Ar dating study undertaken at Berkeley⁹. This single pumice lump contained dark, isotropic glass and abundant euhedral feldspar crystals. The pumice samples were crushed and carefully digested in a mixture of concentrated HF and concentrated H₂SO₄ for 7 d (ref. 16). This digestion process dissolved most of the rock leaving a concentrate of zircon which was further purified by conventional mineral separation techniques. The zircons consist of two distinct varieties: ~95% are colourless, water-clear unzoned euhedral crystals, 200–50 μ m in length and often containing many randomly orientated acicular inclusions together with irregularly shaped fluid inclusions. These crystals all have very low spontaneous fission-track densities. From the perfect, euhedral nature of these zircons, they must be regarded as juvenile crystals, primary to the eruption of the enclosing pumice. Approximately 5% of the zircons are variable in form and much darker in colour. They are light brown to red, often strongly zoned and have a very rounded form, with no discernible crystal faces or inclusions. Since these crystals all have high spontaneous fission-track densities, they appear to be old, detrital zircons and therefore they are excluded from the determinations shown in Table 1.

Four determinations were carried out on sample FMA 517, three at Birkbeck College, London, and one at the US Geological Survey, Denver, Colorado. One determination was carried out on sample ER 74/131 at Birkbeck College. The techniques used for dating the zircons in both laboratories are essentially those described by Naeser¹², except that the tracks were etched in a molten eutectic of KOH and NaOH at 220 °C for between 60 and 100 h (ref. 13). The external detector method was used, ²³⁸U spontaneous fission tracks being counted in the zircon and ²³⁵U induced fission tracks counted in a calibrated external mica detector. Neutron irradiations were carefully monitored by counting tracks from the NBS standard glass SRM 612, which had been calibrated previously against the NBS dosimeter glass SRM 962 and a large number of cobalt wire activation measurements. Using these techniques and a value for the ²³⁸U spontaneous fission decay constant, λ_1 of 6.85×10^{-17} yr⁻¹ (ref. 10) we have obtained ages on standard zircons which agree very closely with their independently known ages¹³. We believe, therefore, that this value of λ_1 is that which is most appropriate to fission-track dating. Fission-track ages have been calculated using the equation of Fleischer *et al.*¹⁴. The results of the five determinations are shown in Table 1.

In addition to the dates shown in Table 1, counting of three of the detrital grains from FMA 517 by one of us (AG) gave dates of 303 ± 32 , 380 ± 62 and 293 ± 52 Myr,

Table 1 Fission-track dating results of two pumice samples from the KBS Tuff, East Rudolf

Sample no. and Determination no.	No. of zircons	Spontaneous tracks No. counted	ρ_s (cm ⁻²)	Induced tracks No. counted	ρ_i (cm ⁻²)	Neutron fluence ϕ (n cm ⁻²)	Age ($\pm 1\sigma$)(Myr)
FMA 517 (1) AJH	10	47	7.05×10^4	1,428	4.28×10^6	2.52×10^{15}	2.55 ± 0.38
FMA 517 (2) AJH	10	56	7.87×10^4	1,129	3.18×10^6	1.59×10^{15}	2.42 ± 0.32
FMA 517 (3) AJWG	6	36	7.09×10^4	802	3.32×10^6	1.81×10^{15}	2.37 ± 0.41
FMA 517 (4) CWN	12	22	9.55×10^4	311	2.70×10^6	1.11×10^{15}	2.41 ± 0.53
						Mean date of FMA 517	2.44 ± 0.08
ER 74/131 (1) AJH	8	21	1.34×10^5	411	5.26×10^6	1.55×10^{15}	2.43 ± 0.55

suggesting an age for the detrital component of about 325 Myr. These results confirm that the pumice blocks had been contaminated by older zircons, possibly during eruption, or by the penetration of detrital grains into the pumice vesicles, during transportation or on deposition. During the fission-track dating procedure this inherited component can be identified easily by the morphology and high track density of these particular zircons. For this reason, it is quite certain that none of these older crystals has been included in the dating of the primary zircons.

The mean of the four determinations on FMA 517 is 2.44 ± 0.08 Myr and this cannot be distinguished from the single determination of 2.43 ± 0.55 Myr for ER 74/131. These dating results provide strong evidence that the primary zircon component in pumice samples from the KBS Tuff in Area 131 has an age close to 2.44 Myr. This may be taken also as the age of eruption and deposition of the KBS Tuff, provided the zircon dates have not been reset by some later event and provided the dated pumices are of the same age as the surrounding tuffaceous matrix. Extrapolation of laboratory annealing studies^{14,16} indicates that fission tracks in zircon should be stable over a period of 3 Myr at temperatures up to at least 300 °C. It is highly unlikely, therefore, that the zircon fission-track dates have been reset by any later thermal events.

Curtis *et al.*⁹ report that sample ER 74/131 has a K–Ar apparent age of 1.82 ± 0.04 Myr whereas Fitch *et al.*⁷ interpret a $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum on FMA 517 as indicating an original date of 2.47 ± 0.60 Myr, overprinted at 1.91 ± 0.03 Myr. To explain such variation in K–Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ estimates of the age of the KBS Tuff, it has been suggested that pumice of more than one age may be present in this horizon⁹. Our limited data do not support this conclusion. The pumice samples ER 74/131 and FMA 517 dated in this study give virtually identical fission-track results of ~2.44 Myr, suggesting that these two samples of pumice cobbles are of the same age.

Conclusion

The mean zircon fission-track date of 2.44 ± 0.08 Myr provides a close estimate of the age of the eruption of the pumice samples collected from the KBS Tuff in Area 131. These fission-track results are also consistent with the palaeomagnetic stratigraphy established by Brock and Isaac¹⁷ on the basis of the original 2.61-Myr date⁴ for the KBS Tuff. From consideration of the data currently available, the date of 2.44 ± 0.08 Myr probably represents a close estimate of the age of eruption and deposition of the KBS Tuff.

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$^{40}\text{Ar}/^{39}\text{Ar}$ dating of the KBS Tuff in Koobi Fora Formation, East Rudolf, Kenya

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$^{40}\text{Ar}/^{39}\text{Ar}$ and conventional K–Ar age determinations on volcanic sanidine–anorthoclase crystal concentrates from pumice lumps contained within the KBS Tuff in the Koobi Fora Formation at East Rudolf indicate an apparent age near 2.42 Myr. Recomputation of data obtained in 1969 from this tuff suggests a more accurate apparent isochron age of 2.42 ± 0.01 Myr for the feldspar, coincident with total fusion conventional K–Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ dates of ~2.4 Myr from the same sample. Age spectra and total fusion dates obtained from other samples of the KBS Tuff confirm this conclusion. Thus, apparent ages of < 2.4 Myr derived from some of our samples and conventional total fusion K–Ar apparent ages in the range 1.6–1.8 Myr obtained from the KBS Tuff by other workers are regarded as discrepant, and may have been obtained from samples affected by argon loss. This latter conclusion is compatible with our sector interpretation of the age spectrum analyses of KBS pumice feldspars. Argument for there being more than one tuff complex or an admixture of older pumice in the KBS Tuff is not supported by our work.

CONTROVERSY over the age of the KBS Tuff in the Koobi Fora Formation of the East Rudolf sedimentary basin of northern Kenya has arisen very largely from apparently conflicting evidence derived from the different approaches to the measurement of geological time. Exploration of the basin in 1968 and 1969 led to the identification of the KBS Tuff and an overlying unconformity^{1–3}. Samples were collected then from an outcrop of this tuff in Area 105, East Rudolf and forwarded to Cambridge for dating⁴. It is now known that the KBS Tuff was deposited by a major river entering Lake Turkana (formerly Lake Rudolf) from the north-east, and numerous lines of evidence suggest that the time between eruption and deposition was negligible^{5–7}. Exploratory conventional K–Ar dating in 1969 revealed the presence of detrital impurities in the vitric tuff sample that made its apparent ages discrepantly high (> 200 Myr). It was immediately clear that unless contamination-free fractions could be found the East Rudolf tuffs would not produce satisfactory samples for geochronometric analysis. Attention was concentrated, therefore, on pumice samples and on a large clean sample of volcanic feldspar extracted from pumice in the field. These

samples were carefully prepared in the laboratory to avoid contamination and analysed by the conventional K-Ar total fusion method and by the then new $^{40}\text{Ar}/^{39}\text{Ar}$ total fusion and age spectrum methods⁸⁻¹¹. Developments in the analytical techniques of $^{40}\text{Ar}/^{39}\text{Ar}$ dating since then¹²⁻¹⁴ enable recomputation of the results obtained, using, in

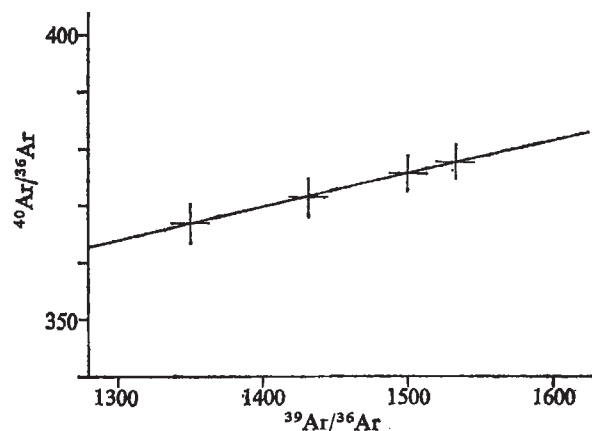


Fig. 1 Isochron diagram for the plateau sector of age spectrum No. 1 (Fig. 1 of ref. 4) of subsample Leakey I/B2 (d). Apparent age of isochron is 2.42 ± 0.01 Myr; intercept value on $^{40}\text{Ar}/^{36}\text{Ar}$ axis is 294.

addition, a more accurate value for the constant of proportionality (J) used in the 1969 experiments. These revised data are listed in Table 1 along with the total fusion K-Ar ages obtained from other subsamples of sample Leakey I for comparison.

Detrital contamination

In all K-Ar dating, some argon loss must be suspected in total fusion results until disproved. In addition, small contamination errors may be present in our total rock pumice samples even though they were selected from the cores of well-washed larger lumps. The concentrates of volcanic feldspar sent to the UK in 1969 consisted of a small number of large (up to 7 mm average diameter) euhedral sanidine-anorthoclase crystals and a greater quantity of small (1.0–5.0 mm average diameter) euhedral feldspar crystals and aggregates containing aegerine-augite and/or iron ore as well as feldspar. A number of different (unequal) subsamples were selected from this feldspar concentrate for dating, so that the presence of any 'detrital' contaminant would be revealed by variation in their apparent ages. The largest, most euhedral feldspars, were hand-picked for $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum analysis No. 1.

Five further subsamples, each consisting of smaller feldspars free from pyroxene and iron ore, were prepared and three of these were subsequently used for total fusion $^{40}\text{Ar}/^{39}\text{Ar}$ and total fusion conventional K-Ar dating.

The virtual coincidence of the apparent ages obtained from determinations made by different methods on four different subsamples of the feldspar concentrate Leakey I/B2 suggests that this concentrate does not suffer from significant detrital contamination. Error attributable to variable but very minor argon loss remains, however, and this is confirmed by the shape of the age spectrum. Thus, the best minimum age for the KBS Tuff currently available from this sample is 2.42 ± 0.01 Myr (revised apparent isochron age of plateau sector of age spectrum No. 1).

Argon loss

During the period 1971–73 further samples were collected from the KBS Tuff at various localities in the East Rudolf basin by I. C. Findlater. Each of the samples was a cleaned and acid-washed feldspar crystal concentrate extracted from

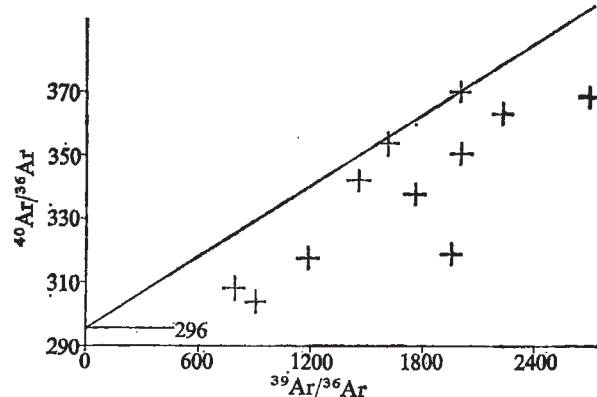


Fig. 2 Correlation diagram plot of total fusion $^{40}\text{Ar}/^{39}\text{Ar}$ age determination data obtained from feldspar concentrates separated from KBS pumice, East Rudolf, Kenya¹⁵. $^{39}\text{Ar}/^{36}\text{Ar}$ values are normalised to a constant J . See text. The straight line is the 2.4-Myr isochron envelope.

pumice by hand picking only the most obviously juvenile crystals from sawn slabs of pumice beneath a binocular microscope. The results of twelve total fusion and six age spectrum $^{40}\text{Ar}/^{39}\text{Ar}$ age determinations on these KBS feldspar samples were briefly summarised at a symposium held in Nairobi in 1973¹⁵. The apparent ages obtained from samples from Areas 10, 130, 131 and 105 show a scatter between 0.5 and 2.4 Myr. The available $^{40}\text{Ar}/^{39}\text{Ar}$ total fusion data from the KBS Tuff pumice feldspars is plotted as a correlation diagram in Fig. 2.

Because of the care taken in their preparation, and by analogy with the data obtained from the various Leakey

Table 1 Conventional K-Ar, new and revised $^{40}\text{Ar}/^{39}\text{Ar}$ dating results from sample Leakey I, KBS Tuff, Area 105, East Rudolf, Kenya ($Q = 0.15152$ GG2 muscovite monitor)

	Total fusion K-Ar dates (Myr)	Total fusion $^{40}\text{Ar}/^{39}\text{Ar}$ dates (Myr)	$^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum dates
Leakey I/B1 four pumice cores, total rock	$3.63 \pm 2.1^*$ 2.40 ± 1.0	$3.12 \pm 1.1^{\dagger\dagger}$ $2.26 \pm 0.5^{\dagger}$	
Leakey I/B2 feldspar crystal concentrate (six independent subsamples: a-f).	(a) 2.38 ± 0.3 (b) 2.37 ± 0.3	(c) $2.39 \pm 0.3^{\dagger}$	(d) 2.42 ± 0.01 Myr. Four point plateau sector analysis of age spectrum No. 1 (see Fig. 1)

*Contamination may have been present in these two pumice total rock samples, but in view of the large experimental errors inherent in their analysis, this cannot be regarded as proven.

† Revision of previously published data.

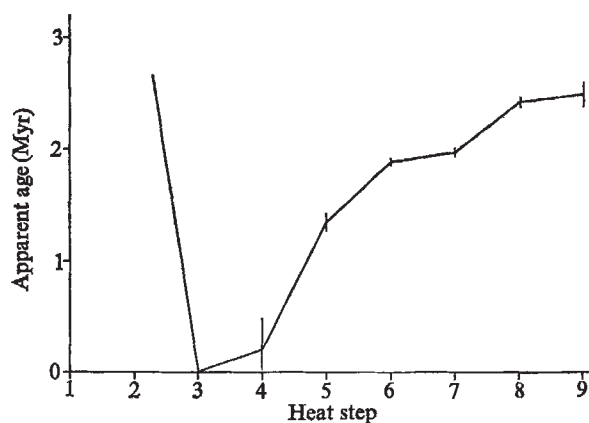


Fig. 3 $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum of KBS pumice feldspar sample FMA 274, Area 10, East Rudolf. The two plateau sectors of the spectrum have apparent ages of 1.94 Myr and 2.46 Myr respectively.

I/B2 feldspar crystal subsamples, the results are very unlikely to be marred by contamination. The scatter of apparent ages obtained must be explained, therefore, by the loss of varying amounts of Ar from the feldspars. If it is accepted that contamination is not significant in these samples, then a minimum age of 2.4 Myr for the KBS Tuff is indicated assuming an intercept value of 296 on the $^{40}\text{Ar}/^{39}\text{Ar}$ axis.

High temperature volcanic sanidine-anorthoclases are highly unstable minerals with aluminium and alkali atoms at random sites in the lattice. During subsequent changes of state towards more ordered low temperature forms of greater stability, significant losses of Ar may result. If these order-disorder processes are accentuated at any time by periods of thermal or hydrothermal activity, then related Ar loss may be identifiable in the K-Ar age patterns of samples of these feldspars. We believe that the complexities of some of the age spectra we have obtained from KBS feldspar samples can thus be explained and may even be resolvable^{3,15}.

Age spectra

Complex age spectra from four samples of KBS pumice feldspar from Area 130, East Rudolf (FMA 201, 206, 225, 227) provide strong evidence of K-Ar age discrepancy^{16,17}, which we attribute to argon loss. Two major age components can be recognised in the four age spectra: one apparently at 1.75–1.8 Myr and another apparently at ~1.07 Myr. When the data defining these sectors are plotted on correlation diagrams, regression line intercept values considerably less than 296 are obtained on the $^{40}\text{Ar}/^{39}\text{Ar}$ axis, thus giving further support to the overprinting hypothesis^{17,18}. Similarly it can be suggested that at least two age sectors, ~1.9 Myr and 1.02 Myr are present in the complex age spectrum obtained from a further sample (FMA 294) of KBS pumice feldspar from Area 105. Such suggestions are not, however, conclusive.

An age spectrum obtained from a sample of clean, detritus-free KBS pumice feldspar (FMA 274) from Area 10, East Rudolf is, however, particularly instructive. Two aspects of the data from this sample are illustrated in Figs 3 and 4. The age spectrum (Fig. 3) is seen to have two short plateau segments apparently ~1.94 Myr and 2.46 Myr respectively. This type of double plateau age spectrum might be interpreted as suggesting either partial overprinting at 1.94 Myr of feldspar originally 2.46 Myr old, or the presence of contamination, but when the data are examined on a correlation diagram (Fig. 4) it is seen that another interpretation is possible: two slightly different feldspar components of unlike initial argon composition

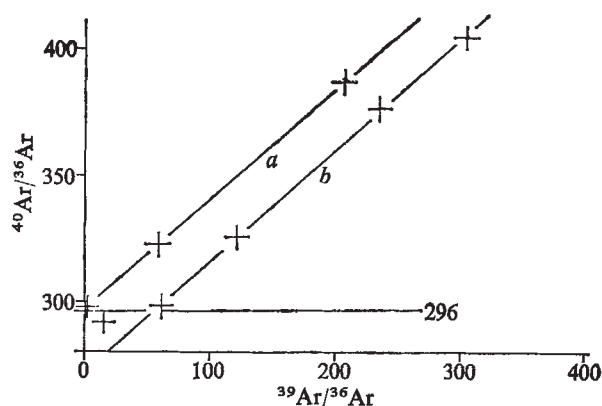
but of identical apparent age are present, one having a regression age of 2.39 ± 0.01 Myr (intercept value, 297) the other 2.42 ± 0.03 Myr (intercept value, 272). The difference in intercept value between these two age components could result from one being accessory feldspar outgassed during the eruptive event of 2.4 Myr ago, whereas the other is of entirely juvenile origin. We feel that the data from sample FMA 274 provide strong additional evidence for a 2.4-Myr age for the KBS Tuff. Thus, our dating of samples of KBS pumice feldspar from other localities at East Rudolf seems to confirm the minimum age of 2.42 Myr derived from sample Leakey I and to suggest that discrepancies from Ar loss, particularly ~1.75–1.9 Myr and 1.05 Myr may be important in many KBS samples (see also the data from sample FMA 517, discussed below).

Opposition to our dating

Over the past five years, opposition to the acceptance of a ~2.5-Myr age for the KBS Tuff has come from three sources: first, archaeologists and palaeoanthropologists disturbed by the consequent antiquity of hominid fossils and stone tools found close to or associated with the KBS Tuff, second, palaeontologists reporting apparent misfits between the faunal sequences at East Rudolf and elsewhere, and third, from a small programme of conventional total fusion K-Ar age determinations on East Rudolf pumice samples undertaken at Berkeley^{19–21}. We believe that the attempts at biostratigraphic and archaeological correlation so far made have been premature and confidently expect the problems raised by these workers^{22–24} to disappear as more accurate faunal lists and local time scales are produced. The inconsistencies that have arisen from the application of different K-Ar dating techniques to samples of the same tuff do, however, require further comment now.

At present, the KBS Tuff is the only tuff in the East Rudolf succession whose apparent age is controversial. For example, from an extensive combination of total fusion and age spectrum $^{40}\text{Ar}/^{39}\text{Ar}$ age determinations, we estimate the best age of the Chari/Karari Tuff level at East Rudolf to be 1.32 ± 0.05 Myr (ref. 6). This particular age was an isochron determination derived from a pumice sanidine sample which we would regard as ideal K-Ar dating material, totally free from either initial argon or argon loss discrepancies. It is instructive that identical ages have been obtained for the Chari and Karari Tuffs by conventional K-Ar dating at Berkeley (G. H. Curtis, personal communication) and similarly from Omo Tuff KNW2,

Fig. 4 Correlation diagram obtained from age spectrum analysis of KBS pumice feldspar sample FMA 274. Two distinct components seem to be present with regression line ages of a, 2.39 ± 0.01 Myr (intercept value: 297) and b, 2.42 ± 0.03 Myr (intercept value 272).



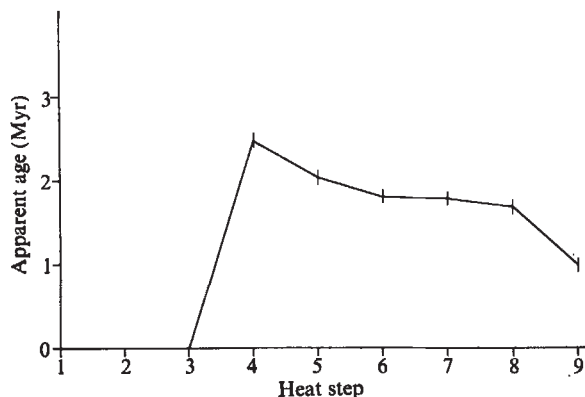


Fig. 5 $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum of KBS pumice feldspar sample FMA 517, area 131, East Rudolf (same locality as Berkeley samples ER74-131). The age spectrum has a 'peak' age of 2.47 ± 0.6 Myr then declines through a 'flat' at ~ 1.8 Myr.

which is thought by Brown²³ to be the lateral equivalent of the Chari/Karari level in southern Ethiopia. Thus, it is clear that the inconsistencies between conventional K-Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ dating of the KBS Tuff most likely arise from some inherent characteristics of the samples rather than differences in the dating methods used.

Conventional K-Ar dates of feldspars from the KBS Tuff quoted by Curtis *et al.*²¹ scatter between 6.90 ± 0.05 Myr and 1.50 ± 0.02 Myr. Clearly, detrital contamination was present in some of the Berkeley dating samples prepared by heavy liquid concentration alone, but, the subsequent recognition of a number of yellow, rounded, and thus probably detrital, grains in the unused portion of a dated sample does not necessarily prove that any detrital grains were present in the small aliquot of the sample actually used for the argon analysis. Thus, it is still possible that some of the apparent ages between 1.85 Myr and 2.4 Myr rejected by Curtis *et al.* may result, at least in part, from the presence of a non-detrital 2.4-Myr-old component.

Comparison

Data obtained at Cambridge from the $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum analysis of KBS pumice feldspar sample FMA 517, collected from the same locality in Area 131, East Rudolf as Berkeley samples ER74-131 are presented in Figs 5 and 6. The mean of six conventional total fusion K-Ar ages on undoubtedly detritus-free pumice feldspar and glass fractions

of the samples ER74-131 carried out at Berkeley was 1.82 ± 0.04 Myr. This age is regarded by Curtis *et al.* as a good estimate for the date of eruption of the KBS Tuff in Area 131, whereas, from our experience we would interpret it as being the date of total argon loss, affecting glass and feldspar crystals alike. Unfortunately, the results of the analysis of sample FMA 517 are not absolutely conclusive. The age spectrum (Fig. 5) contains a 'peak' apparent age of 2.47 ± 0.6 Myr, but at later steps the apparent age curve declines to a 'flat' apparently close to 1.8 Myr. On the correlation diagram (Fig. 6) the data fall into two groups: the younger of the two age components has an apparent age of 1.91 ± 0.03 Myr (with an intercept value of 285, suggestive of overprinting). Because insufficient data are available, the apparent age of the older component cannot be estimated with any more precision than was given to it by the peak on the age spectrum. The major feldspar component in sample FMA 517 clearly has an apparent age of 1.91 ± 0.03 Myr.

The six total fusion K-Ar ages obtained at Berkeley, the apparent age of the lower sector of the age spectrum, and a total fusion $^{40}\text{Ar}/^{39}\text{Ar}$ apparent age of 1.72 ± 0.11 Myr obtained from another subsample of pumice feldspar FMA 517 at Cambridge, must be regarded as less precise estimates of the age of this component, because they undoubtedly contain an uncorrected initial argon discrepancy and, in the case of the total fusion dates, a variable contribution from the older feldspar component.

Conclusion

By analogy with our other work on the KBS Tuff, we believe that in Area 131 the older feldspar age component represents the true age of crystallisation and the dominant younger component defines no more than the age of a widespread overprinting event, but there is nothing in the available K-Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ data that makes this interpretation unique.

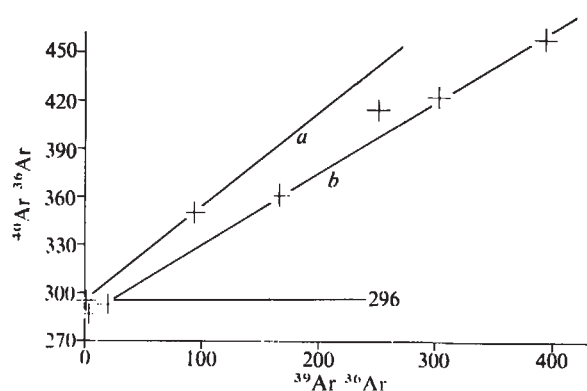
In these circumstances we must await confirmation from further work on samples from Area 131, especially work using methods of estimating geological time which are independent of the K-Ar dating technique. At East Rudolf, palaeomagnetic reversal chronology^{26,27}, and fission track dating^{28,29} are the methods most likely to resolve this controversy.

One further conclusion appears inescapable: as multiple age component systems and age components with apparent initial Ar isotope ratios unlike the modern atmosphere ratio are present amongst the pumice feldspar samples from the KBS Tuff, variation in the total fusion K-Ar or $^{40}\text{Ar}/^{39}\text{Ar}$ apparent ages of samples from different localities is to be expected. Thus the conventional total fusion K-Ar dating evidence at present available cannot be used either to confirm or to deny the presence of more than one tuff or pumice population at the KBS level. It is our opinion, however, that the balance of evidence from a combination of the currently available K-Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ work strongly suggests that only one tuff complex is present, it was deposited within a short interval in all areas, it contains one dominant pumice generation and this pumice is 2.42 Myr old.

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Fig. 6 Correlation diagram obtained from age spectrum analysis of KBS pumice feldspar sample FMA 517. Two age components seem to be present. The age of the older (a) cannot be defined more closely than the rather imprecise 'peak' age of 2.47 ± 0.6 Myr., but the younger (b) has a regression age of 1.91 ± 0.03 Myr (intercept value, 285).



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Cloned synthetic *lac* operator DNA is biologically active

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A chemically synthesised duplex DNA fragment containing the sequence of the lac operator was cloned in E. coli using the vehicle pMB9. Clones containing lac-pMB9 hybrid DNA produced β -galactosidase constitutively and the hybrid DNA bound the lac repressor specifically.

THE nucleotide sequence of the *lac* operator has been determined by Gilbert and Maxam¹. We have synthesised chemically an oligonucleotide duplex, 21 nucleotides long and containing the sequence of the *lac* operator, and have shown that it binds the *lac* repressor specifically². Here, we report the enzymatic joining of a synthetic *lac* operator fragment with protruding 5' pA-A-T-T sequences (Fig. 1) directly to *EcoRI* endonuclease-cut pMB9 DNA³. After transformation, clones containing hybrid *lac*-pMB9 DNA expressed the biological activity *in vivo* of the chemically synthesised *lac* operator by producing β -galactosidase constitutively. Furthermore, the *lac* operator DNA reisolated from the hybrid DNA by cleavage with the *EcoRI* restriction enzyme⁴ showed specific and strong binding to the *lac* repressor. The reisolated *lac* operator DNA with protruding 5' pA-A-T-T sequences can be joined by T4 polynucleotide ligase to produce multi-operator fragments of up to 18 copies of the *lac* operator in tandem. Some of the multiple operators were ligated with *EcoRI* endonuclease-cut pMB9 DNA, and hybrid DNA with two copies of the *lac* operator was isolated.

In addition to the specific method reported here, a more general approach for inserting any even-ended DNA fragment into plasmid DNA has been developed. In this approach⁵, a chemically synthesised decanucleotide duplex containing *BamI* restriction endonuclease⁶ recognition sequence is joined to an even-ended *lac* operator DNA duplex² by T4 polynucleotide ligase (ref. 6). Digestion of the ligated product with the *BamI* endonuclease generated the protruding 5' pG-A-T-C sequence. This molecule was then joined to the *BamI* endonuclease-cut pMB9 DNA. Details of these results will be reported elsewhere¹⁰.

Characterisation and analysis of hybrid plasmids

Results in Table 1 show that clones 50, 67 and 162 produced large amounts of β -galactosidase and that the DNA isolated from these clones bound the *lac* repressor extensively. The DNA from pMB9 plasmid and the three clones which produced very low levels of β -galactosidase all showed essentially no binding. The DNA from clones 67 and 162 was further examined by digesting the samples with *EcoRI* enzyme and carrying out electrophoresis using a 3%:15% polyacrylamide step gel. To detect *lac* operator-size small DNA fragments, the digest was labelled by repair synthesis⁷ at the cohesive ends with DNA polymerase I and α -³²P-dATP. Figure 2 shows that the plasmid-size linear DNA band is clearly present in all cases. However, digests of the DNA from clones 67 and 162 also show an operator-size DNA fragment in the lower portion of the gel. The ratio of counts in the operator fragment to the plasmid DNA was nearly 1:1 for clones 67 and 162, indicating that only one copy of the *lac* operator had been inserted per plasmid. This was explored further by partially digesting the hybrid plasmids with the *EcoRI* enzyme followed by labelling, using repair synthesis (Fig. 3). Even though partial digestion products of the plasmid DNA can be seen, only one operator-size species is present in all cases. If multiple operator species were present, one would expect to see one or more bands above that of the *lac* operator DNA in the 15% gel. The small fragment was shown to be *lac* operator DNA on elution from the gels and testing its binding to the *lac* repressor. The results are shown in Table 1b. Each fragment tested gave excellent binding with the *lac* repressor. Since only the centre 21 nucleotides out of the completely repaired 31-nucleotide-long duplex had the correct sequence for the natural *lac* operator, the nucleotide sequences beyond the central 21-nucleotide sequence were not needed for the recognition by the *lac* repressor². In fact, wrong sequence beyond the central 21 nucleotides did not seem to interfere with the repressor binding.

The structure of the operator fragments was confirmed by determining their nucleotide sequence. Operator fragment was labelled at the 3'-ends with ³²pA³²pA by partially repairing the protruding 5'pA-A-T-T ends using DNA polymerase I and α -³²P-dATP. The resulting 29-nucleotide-long strands were

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and 5' . . . T-T-A-T-C-C-G-C-T-C-A-C-A-A-T-T-G-³²pA³²pA, respectively. Written in the duplex form, these sequences overlap by nine nucleotides which confirm the expected sequence of the two 27-nucleotide-long single strands as shown in Fig. 1b.

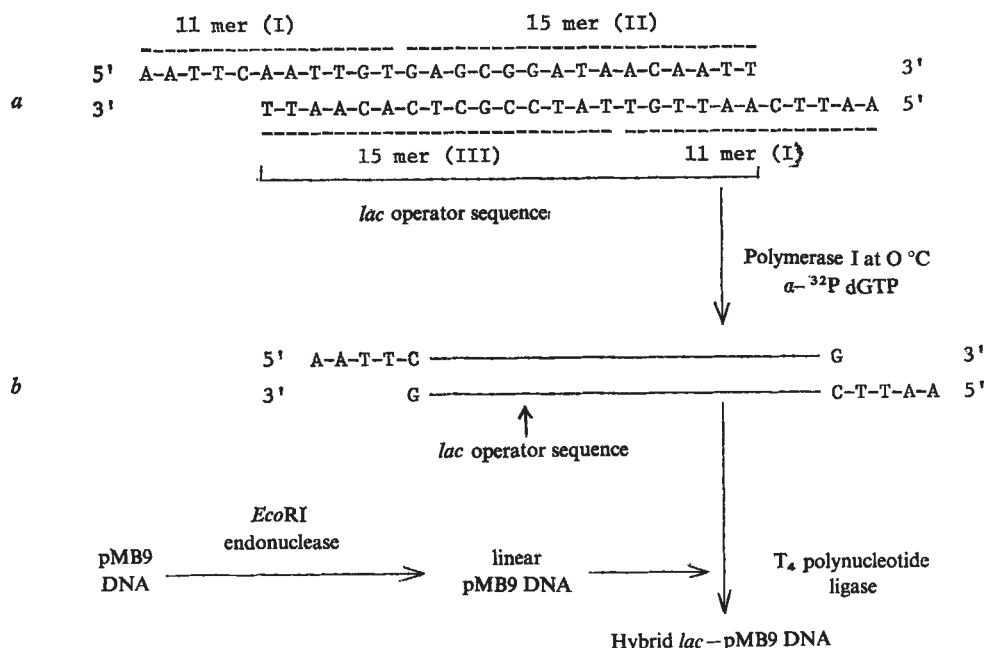


Fig. 1 Joining of the chemically synthesised *lac* operator duplex with protruding 5' A-A-T-T sequence with *EcoRI* endonuclease-cut pMB9 DNA. The three oligonucleotides used to make the duplex fragment (structure *a*) were synthesised by the modified phosphotriester method¹¹⁻¹⁴ and are indicated by the dotted lines. Enzymatic joining of the oligonucleotides was carried out with T4 polynucleotide ligase² and the duplex fragment was separated from the single-stranded oligonucleotides by elution from a Sephadex G-75 column with 0.1 M NaCl. The duplex (underlined with solid line) was synthesised so that the recognition sequence of the *EcoRI* restriction endonuclease was present at both ends. Before joining the fragment with pMB9 DNA it was necessary to incorporate one dGMP residue into each of the single-stranded ends to produce structure *b*. This *lac* operator duplex with protruding 5' A-A-T-T sequence (1 pmol) was joined to pMB9 DNA (0.3 pmol) which had been made linear by digestion with *EcoRI* endonuclease. Ligation reaction was at 12.5 °C for 20 h. The resulting hybrid *lac*-pMB9 DNA was used to transform 3×10^7 competent *E. coli* HB129 cells³ as follows: The DNA and recipient cells were mixed together and incubated at 0 °C for 30 min. The temperature of the mixture was raised to 42 °C for 2 min and then chilled to facilitate uptake of the DNA by the cells. Nine volumes of prewarmed L-broth were added and the cells allowed to recover at 37 °C for 2 h. One volume of L-broth supplemented with 10 µg ml⁻¹ of tetracycline was then added. After an additional 30 min at 37 °C the tetracycline concentration was brought up to a final level of 20 µg ml⁻¹. This cell suspension was used to inoculate 100 ml of M9 medium for the isolation of a larger amount of plasmid DNA¹⁵. This plasmid DNA (30 µg) which contained some hybrid *lac*-pMB9 DNA was enriched for *lac* sequences by binding it to the *lac* repressor (4.5 µg) on a Millipore filter, and eluting it with 1 ml of 1 mM isopropyl thiogalactoside (IPTG). This DNA (6% of the input), enriched for *lac* sequences, was used for a subsequent transformation on nutrient agar plates containing 20 µg ml⁻¹ of tetracycline. The frequency of transformation was 6.4×10^{-4} transformants per µg per DNA per viable cell whereas in the same conditions native pMB9 gave a frequency of 1.8×10^{-5} .

<i>a</i>	Clone	β-Galactosidase level	Binding (%)	<i>b</i>	Clone	Binding (%)
	44	Low	0		67	28
	50	High	70		162	31
	67	High	72			
	84	Low	4			
	114	Low	3			
	162	High	60			
	pMB9	Low	0			

Lac repressor binding of hybrid *lac*-pMB9 plasmids and isolated operator fragments. All *lac* repressor binding assays were performed in 100 μ l of binding buffer¹⁷ containing 1.3 μ g of *Hind*II + III digested λ plac5 DNA (40 fmol *lac* operator), radioactive DNA sample (1–4 fmol) and 200 fmol *lac* repressor². The mixture was left at room temperature for 30 min. Triplicate samples of 30 μ l each were filtered through Millipore HAWP 0.45- μ m filters and then washed three times with 200 μ l of a washing buffer¹⁷. 1 ml of 1 mM IPTG was then passed through the filter and the eluate collected. Percentage binding is defined as the fraction of total input counts eluted from the filters with IPTG.

a, Assay for β -galactosidase level was carried out as follows. Plasmid pMB9 is a derivative of plasmid colicin E1 (ref. 3) and, therefore, in the unamplified state, exists in 10–20 copies per cell.

Because there are only about 10 molecules of *lac* repressor per cell, clones containing the *lac* operator ligated to plasmid pMB9 should have all *lac* repressor molecules bound to the *lac* operator on plasmids, leaving the *E. coli lac* operon derepressed, thus producing β -galactosidase constitutively. Clones were grown up in 1 ml L-broth containing tetracycline and were screened for β -galactosidase escape synthesis. 0.1 ml of the overnight cultures was diluted to 1 ml, and the cells were lysed with one drop of CHCl_3 and 0.1% sodium dodecyl sulphate. *O*-nitrophenyl- β -D-galactoside (ONPG) was added to a final concentration of 0.8 mg ml^{-1} . In this qualitative assay¹⁶, a positive indication of constitutive β -galactosidase production was the formation of a yellow colour characteristic of the action of β -galactosidase on ONPG. Of the 180 clones tested, three showed high levels of β -galactosidase synthesis. DNA (1 μg) from six clones (including three clones as controls) was labelled by nick translation¹⁸ with DNA polymerase I (7 units) in 100 μl of a mixture containing 50 mM Tris-HCl (pH 7.9), 50 mM KCl, 10 mM dithiothreitol, 10 mM MgCl_2 , and 8 μM of the four dNTPs (including α -³²P-dGTP, 15 Ci mmol^{-1}). After 2 h at room temperature, the reaction was terminated by ethanol precipitation. Input c.p.m. in the binding assay was between 500 and 1,500 c.p.m. Percentage binding had been corrected for background, determined by results without the addition of the repressor. Background was between 3–6% of the total input counts, *b*, Operator fragment eluted from a gel⁷ as in Fig. 2 was found with the repressor as described earlier¹⁷. Input c.p.m. was between 250 and 600 c.p.m. Percentage binding as shown had been corrected for background which was 2–4%.

Fig. 2 An autoradiograph of gel electrophoresis of hybrid *lac*-plasmids digested with the *Eco*RI enzyme and labelled by repair synthesis⁷. The hybrid *lac*-pMB9 DNA was isolated after amplification by the addition of chloramphenicol to the bacterial culture¹⁸. The hybrid plasmid DNA (1 μ g) was digested with the *Eco*RI enzyme in 20 μ l of a solution containing 50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM MgCl₂ and 3 units of the enzyme (one unit completely digests 1 μ g of DNA at 37 °C in 1 h) at 37 °C for 30 min. The reaction was terminated by chilling to 0 °C. The cohesive ends generated by the *Eco*RI enzyme were then repaired by bringing the mixture to a volume of 30 μ l containing 70 mM KPi (pH 7.0), 10 mM dithiothreitol, 2 μ M α -³²P-dATP and dTTP (110 Ci mmol⁻¹), and 3 units of DNA polymerase I. After 2 h at 0 °C the reaction was terminated by the addition of 1 volume of 1 M Tris-HCl, followed by two volumes of ethanol. Samples were electrophoresed on a composite slab gel of 3% and 15% polyacrylamide (acrylamide-*bis*-acrylamide = 20:1) with dimensions of 20 $\times$ 40 $\times$ 0.3 cm. The running buffer was 50 mM Tris-acetate (pH 7.9). Xylene cyanol FF (XC) and bromophenol blue (BPB) were used as tracking dyes. Electrophoresis was at 150 V for 16 h.

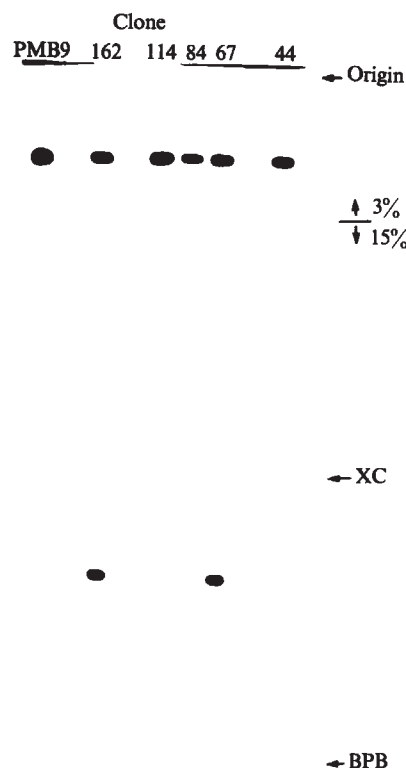
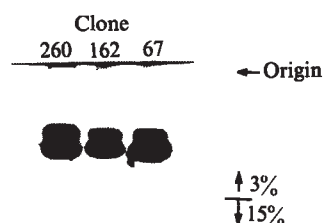


Fig. 3 An autoradiograph of gel electrophoresis of hybrid plasmids partially digested with the *Eco*RI endonuclease and labelled by repair synthesis. The procedures were the same as those given in the legend for Fig. 2, except that 1 unit of the *Eco*RI enzyme and 3 min of incubation were used.

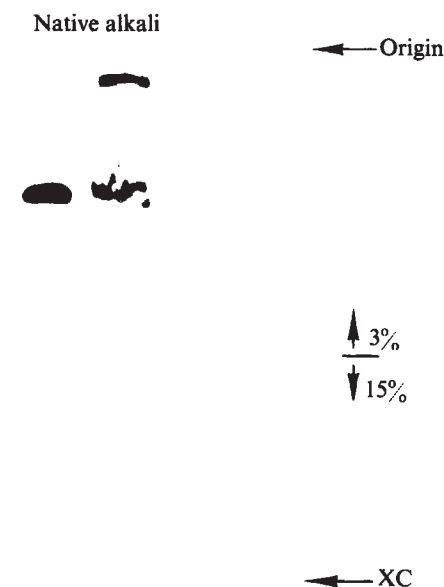
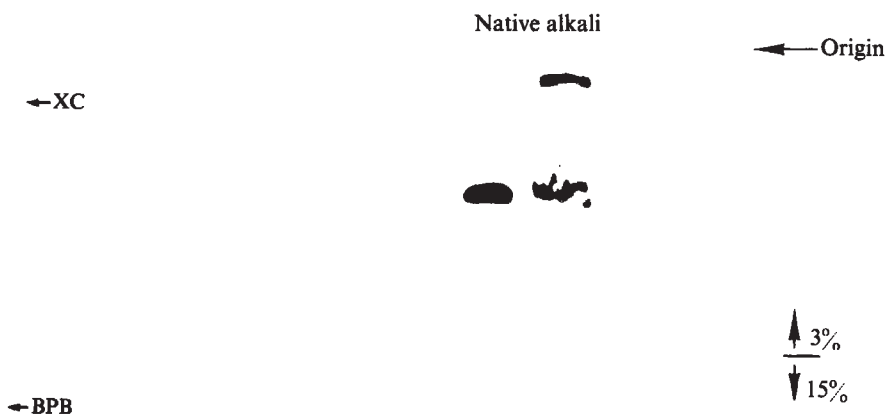


Fig. 4 Strand separation of the *lac* operator duplex. DNA from clone 67 was digested with the *Eco*RI enzyme and labelled by partial repair synthesis by DNA polymerase I using only α -³²P-dATP. This now yields DNA fragments with single-strand length of 29 nucleotides. After ethanol precipitation one half the material was dissolved in standard loading buffer and the other half was dissolved in 0.3 N NaOH-10% glycerol. Bromophenol blue and xylene cyanol FF were added to the alkali sample just before loading on a 20 $\times$ 40 $\times$ 0.3-cm 3%:15% step gel of polyacrylamide (acrylamide-*bis*-acrylamide = 20:1). The gel was run in Tris-borate buffer (pH 8.3) at 400 V. Two distinct bands corresponding to the separate strands of the *lac* operator fragment can be seen in the slot containing the alkali sample. This can be compared with the mobility of the native fragment which is in the other slot. These bands were eluted and used for sequence analysis as described in the text.



Fig. 5a and b, Autoradiographs of two-dimensional electrophoresis-homochromatography of partial DNase digests of the separated strands of the *lac* operator fragment reisolated from hybrid plasmid DNA as in the legend for Fig. 2 and 4. The sequences were deduced by the quantitative mobility-shift analysis⁹. The sequence of 17 nucleotides from one strand and 19 nucleotides from the other strand overlaps by 9 nucleotides to give the sequence of the two 29-nucleotide-long single strands which confirms that 27-nucleotide long sequence as shown in Fig. 1b

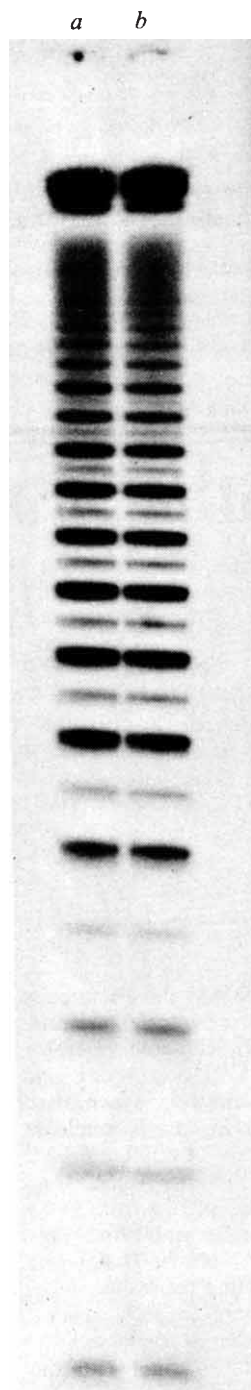
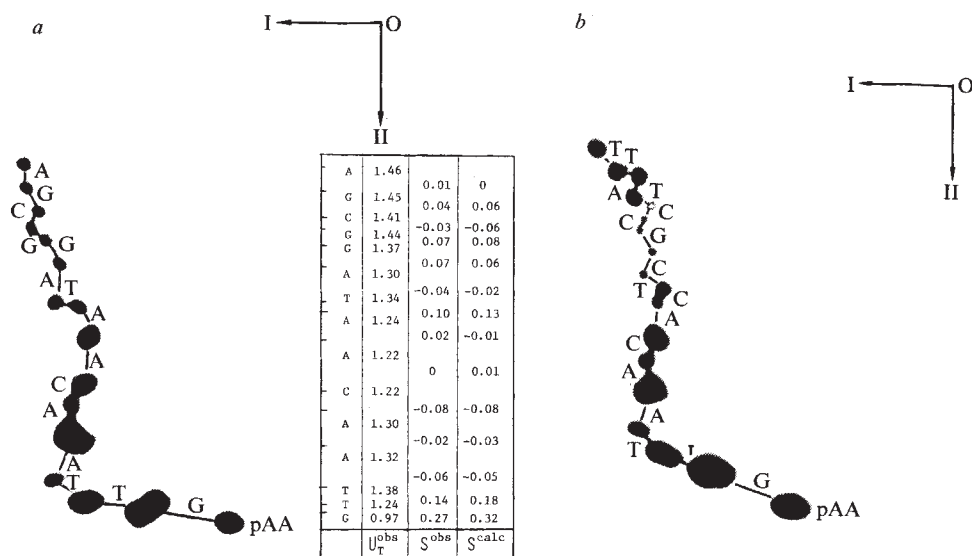


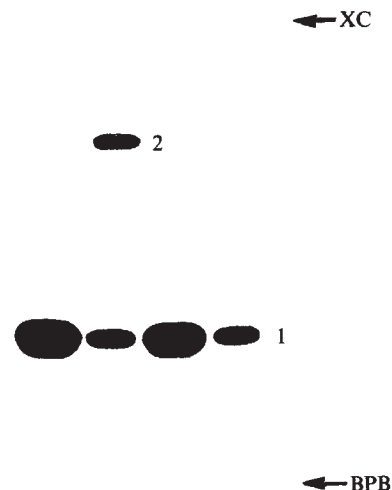
Fig. 6 An autoradiograph of gel electrophoresis of polymeric *lac* operators. Cloned *lac* operator fragment reisolated from hybrid *lac*-pMB9 DNA by *Eco*RI enzyme digestion was labelled at the 5' end with ³²P and polynucleotide kinase⁹. The fragment was eluted from a gel and then joined end-to-end with itself (*lac* operator DNA concentration was 130 ng ml⁻¹, using T4 polynucleotide ligase at 12.5 °C. The reaction mixture was analysed on a 10% polyacrylamide gel with a 3% stacking gel, using 40 mM Tris-borate (pH 8.3) electrophoresis buffer. When no ligase was used (result not shown), only the monomeric operator (band 1) was present. The lighter bands in between dark bands are presumably circular oligomeric and polymeric *lac* operators.

Dimer Monomer ← Origin



3%
10%

Fig. 7 Characterisation of a plasmid containing two copies of the *lac* operator in tandem. The band corresponding to a dimer of the *lac* operator fragment was eluted from the gel shown in Fig. 6. This material was then ligated with *Eco*RI endonuclease-cut pMB9 DNA and used to transform HB129 cells. β-galactosidase constitutive clones were identified by their blue colour on X-gal plates¹⁶. The number of copies of *lac* operator DNA contained in any particular clone was determined by (1) partially digesting (lane p) the hybrid plasmid DNA with *Eco*RI endonuclease, and (2) by complete digestion (lane c). The digest was then labelled by repair synthesis using α-³²P-dATP and DNA polymerase I. The results were analysed by electrophoresis on a 20×40×0.3 cm 3%:10% step gel of polyacrylamide (acrylamide-bis-acrylamide, 20:1) run in Tris-borate buffer. DNA from clone 67 (marked monomer) are shown for comparative purposes. For experiments with complete *Eco*RI endonuclease digestion, the radioactive DNA bands were cut out from the polyacrylamide gel and counted for ³²P. In the dimeric hybrid, *lac*₂-pMB9 DNA (marked dimer), the ratio of counts in the operator fragment (band 1 in the 10% gel) to the plasmid DNA (the dark band above the 3%:10% junction) was close to 2:1 (60,000 c.p.m.:29,500 c.p.m.). In the monomeric hybrid (marked monomer) the ratio of counts was 1:1.



Since the yield of *lac* operator from the *lac*-pMB9 hybrid is relatively low, it will be necessary to produce a plasmid with multiple copies of the *lac* operator. To this end, we attempted to determine whether the cloned *lac* operator fragment can be ligated to itself to form polymeric *lac* operators before ligation to the plasmid DNA. Figure 6 shows that one can clearly distinguish on this polyacrylamide gel multi-operator DNA bands which contain up to 18 copies of the *lac* operator (joined together by the protruding sequence 5'pA-A-T-T). These multi-operator DNA molecules appeared to be linear instead of circular in structure as reflected by the percentage of ³²P counts released by treating each DNA band (up to the nonamer) with bacterial alkaline phosphatase. A dimeric *lac* operator fragment has been inserted into plasmid pMB9 (Fig. 7). When the hybrid *lac*₂-pMB9 DNA was partially digested with *Eco*RI endonuclease a monomeric (band 1) and dimeric (band 2) *lac* operator were found. Insertion of oligo-operator DNA fragments into the plasmid and the characterisation of these hybrid molecules are in progress.

Potential of this technique

The technique described here provides a way of producing large amounts of homogeneous and biologically active *lac* operator DNA for physicochemical studies. The first method (Fig. 1) can also be used to insert any similarly constructed synthetic DNA into a cloning vehicle. The second method¹⁹ which involves end-to-end joining of an even-ended DNA to a synthetic DNA duplex containing the *Bam*I (or other) restriction endonuclease recognition sequence can be used to insert any even-ended DNA molecules into a cloning vehicle. Our technique serves as an example of a general approach in

introducing a *lac* operator in front of a gene or a group of genes, so that the gene can be turned on or off by *lac* repressor with or without IPTG.

K. J. M., thanks Kathe Meyerhoff and Joe Calvo for guidance in the proper use of the transformation procedure. This work was supported by grants from the NIH, the NSF, and the National Research Council of Canada. Heyneker *et al.*¹⁰ have independently succeeded in inserting a slightly different *lac* operator segment into pMB9 plasmid, and their work is reported in the accompanying manuscript.

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Synthetic *lac* operator DNA is functional *in vivo*

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Blunt end ligation with an EcoRI octanucleotide "linker" was used to construct a plasmid containing chemically synthesised lac operator DNA. This plasmid renders the host bacterium constitutive for β-galactosidase.

THE specific binding of the *lac* repressor protein to the *lac* operator, a defined nucleotide sequence in the DNA of *Escherichia coli*, controls the expression of the nearby structural genes of the *lac* operon¹. The nucleotide sequence in the region of the *lac* operator is known^{2,3} and recently the *lac* operator (21 nucleotide base pairs, see Fig. 1) has been synthesised by a phosphotriester chemical method^{4,5}. This chemically synthesised *lac* operator DNA functions *in vitro*, as evidenced by repressor binding activity^{6,7}.

To examine the *in vivo* properties of a chemically synthesised genetic element and to have a convenient con-

tinuous source of a small *lac* operator DNA, the chemically synthesised *lac* operator was cloned in a bacterial plasmid. The scheme which produced an easily clonable operator DNA fragment is depicted in Fig. 1. Using blunt end ligation by T4 DNA ligase⁸, a chemically synthesised octadeoxyribonucleotide containing the *Eco*RI endonuclease recognition sequence⁹ was joined to the 21-nucleotide *lac* operator sequence. The resulting RI-operator DNA fragment was then inserted into the plasmid pMB9 (ref. 9) by using the *Eco*RI cohesive termini and established procedures¹⁰. The pMB9 plasmid was used because it has one *Eco*RI restriction site, carries a tetracycline resistance gene, and can be amplified by chloramphenicol to yield several thousand copies per cell¹¹.

The covalently joined molecules of *lac* operator and *Eco*RI substrate which we refer to as the RI-operator fragment are totally chemically synthesised DNA molecules. The synthesis of β-galactosidase is constitutive in cells

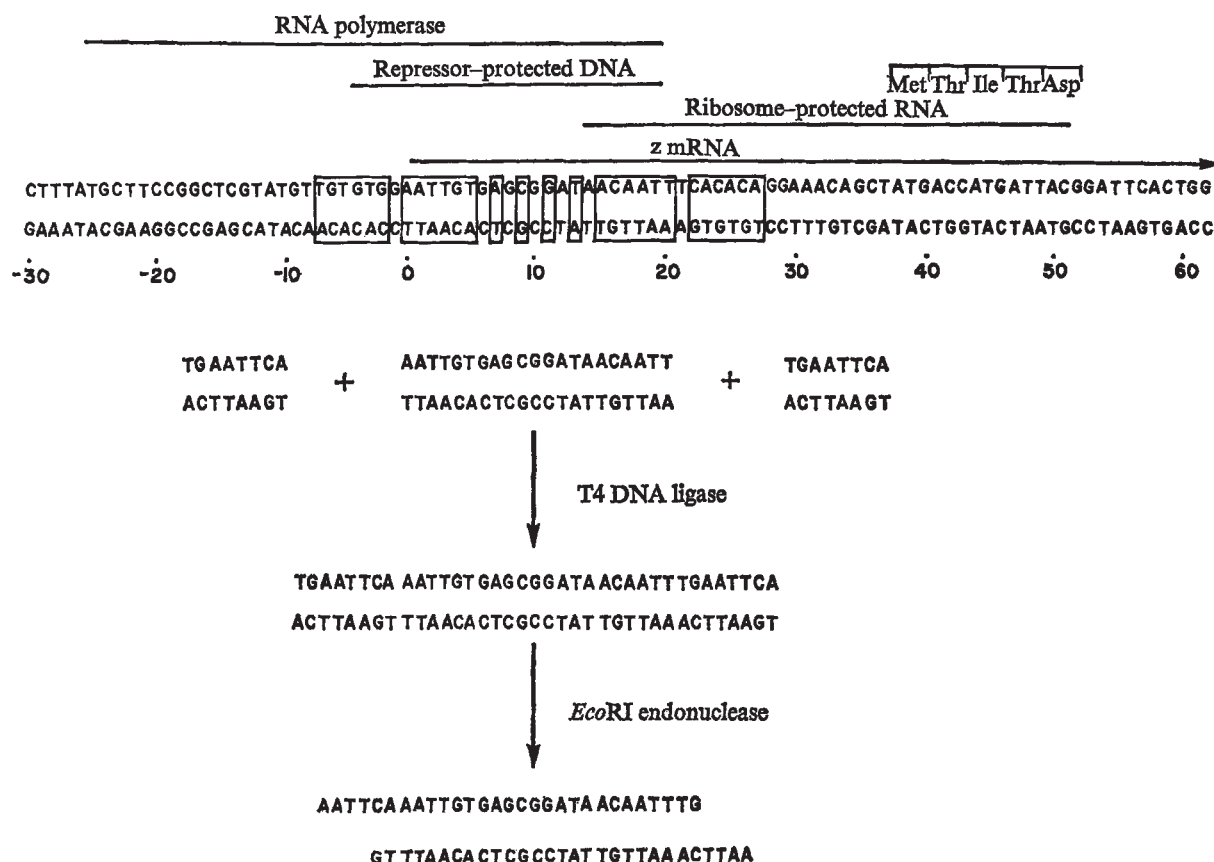


Fig. 1 Experimental plan for cloning of the *lac* operator. The nucleotide sequence at the top of the figure represents most of the control region of the *lac* operon. The 5' termini are on the left of the upper strands as depicted here. The sites for RNA polymerase binding, mRNA initiation, and so on are depicted³. The *lac* operator, where repressor binds, is easily identified by the boxed regions of symmetry. The central operator, where all operator-constitutive mutations occur, is from nucleotide 0 to +20. The extended operator noted by Dickson *et al.*³, is from -7 to +27. The second line shows the nucleotide sequences of the chemically synthesised DNAs. The 21-nucleotide central operator was synthesised by a phosphotriester method^{4,5,18} and the octanucleotide *Eco*RI substrate was made by phosphodiester synthesis⁸. The chemically synthesised DNAs were phosphorylated by polynucleotide kinase, joined by T4 DNA ligase and treated with *Eco*RI endonuclease to produce cohesive ends. The *Eco*RI endonuclease-treated fragment was cloned in the *Eco*RI site of the plasmid pMB9 cloning vehicle⁹. Additional details are given in the legend of Fig. 2.

carrying the RI-operator-pMB9 plasmid because there are about 30 copies of this plasmid per cell in normal growth conditions⁹. The extra operators titrate the 10 to 20 repressor molecules per cell¹², so the single *lac* operon in the host chromosome is not repressed. Therefore, the experiments reported here demonstrate that the genetic constitution of a cell can be altered by the incorporation of chemically synthesised DNA.

The technique devised for cloning the *lac* operator DNA utilises the chemically synthesised octadeoxyribonucleotide containing the substrate for the *Eco*RI endonuclease as a "linker". The addition of short restriction site "linkers" to DNA of any source provides novel flexibility to cloning technology.

Blunt end ligation

Following the procedure given in the legend of Fig. 1, about a 20-fold molar excess of unlabelled *Eco*RI octadeoxyribonucleotide was added to the 5' ³²P-labelled *lac* operator DNA and treated with T4 DNA ligase at 12.5 °C, a temperature slightly below the *T_m* of the *Eco*RI octanucleotide duplex structure⁸. The *Eco*RI linker was joined to the 21 base pair operator DNA by the blunt-end DNA joining activity of T4 DNA ligase, a property of this enzyme discovered by Sgarbetta and Khorana⁷ and recently used by W. Gilbert (personal communication) for plasmid construction.

About 50% of the 5'-terminal phosphates on the *lac*

operator DNA were resistant to alkaline phosphatase after a 3-h incubation. A sample of this reaction, along with separate samples of terminally-labelled *lac* operator and *Eco*RI substrate treated separately with T4 DNA ligase, were subjected to electrophoresis on a 12% polyacrylamide gel. An autoradiogram of the gel reveals that the *lac* operator DNA and the *Eco*RI DNA were joined (Fig. 2); the ligated samples of the *Eco*RI linker and the combined *Eco*RI linker plus *lac* operator had molecules of higher molecular weight which must have been formed by the action of the T4 DNA ligase. *Eco*RI endonuclease digestion eliminated the larger molecules, and a new oligonucleotide, 29 nucleotide base pairs in length, appeared.

Cloning

The RI-operator DNA produced by *Eco*RI endonuclease digestion was ligated to *Eco*RI endonuclease-treated pMB9 plasmid DNA and used to transform *E. coli* RR1, which has an *i⁺z⁺* genotype⁹. After 3 h growth for expression of tetracycline resistance, samples of the culture were plated on minimal medium containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and tetracycline. X-gal is not an inducer for the *lac* operon but is a substrate for β -galactosidase^{13,14}. After 48 h incubation at 37 °C, 0.3% of the tetracycline-resistant colonies were blue, indicating that these colonies were constitutively synthesising β -galactosidase, and releasing the blue dye 5-bromo-4-chloro-indigo. Since there are about 30 copies of the pMB9 plasmid per

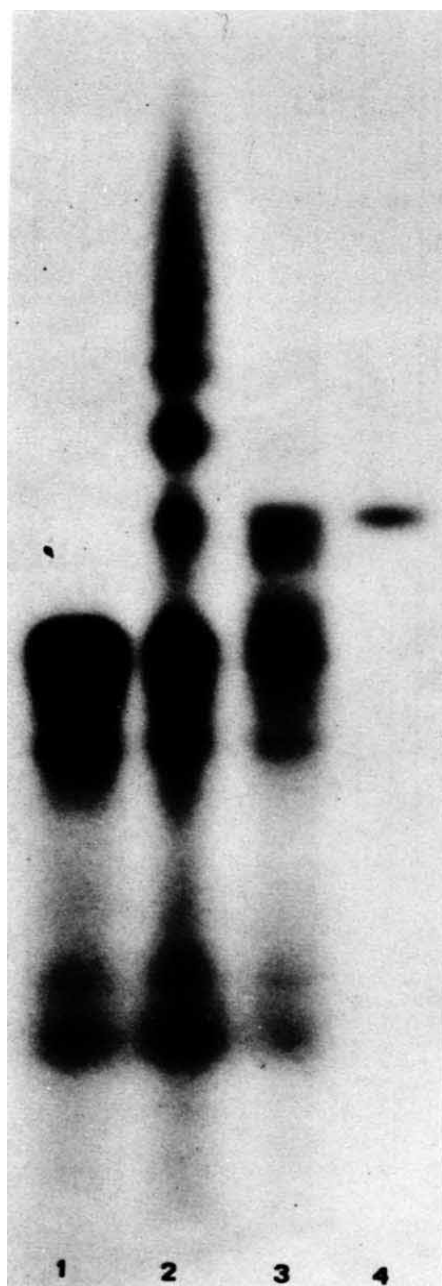


Fig. 2 Blunt end ligations and cloning. The chemically synthesised *lac* operator DNA was labelled at the 5' termini by treatment with T4 polynucleotide kinase (2–5 units per 100 pmol of 5' ends) in a reaction containing 50 mM Tris-HCl, pH 9.5, 10 mM MgCl₂, 5 mM dithiothreitol, 5% glycerol and 2 μ M γ -³²P-ATP (1,000 Ci mmol⁻¹). After 1 h incubation at 37 °C, additional T4 kinase and ATP (to 100 μ M final concentration) were added to the reaction and incubation was continued for an additional 30 min to obtain near quantitative phosphorylation of the DNA. The labelled DNA was separated from residual ATP by chromatography on Sephadex G-50. 75 ng of ³²P-labelled 21-nucleotide *lac* operator DNA was joined with 550 ng of unlabelled *Eco*RI linker DNA by treatment with 0.4 units of T4 DNA ligase¹⁹ in a 10- μ l reaction volume (20 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM dithiothreitol, and 0.4 mM ATP). The reaction was incubated for 3 h at 12.5 °C. For cloning, a sample of the ligated mixture of *Eco*RI linker DNA and operator DNA (about 2.5 pmol of operator) was combined with 0.2 pmol of pMB9 DNA and treated with 50 units of *Eco*RI endonuclease⁸ at 37 °C for 60 min in 30 μ l (final volume) of 100 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, and 0.02% NP40. After inactivation of the *Eco*RI endonuclease by chloroform extraction, 5 μ l of 10-times concentrated ligase buffer (0.66 M Tris-HCl, pH 7.6, 66 mM MgCl₂, 0.1 M dithiothreitol, and 4 mM ATP) and 0.4 units of T4 DNA ligase were added and the volume adjusted to 50 μ l. The reaction was incubated for

cell and only about 10 *lac* repressor molecules, synthesis of β -galactosidase becomes constitutive if the plasmid contains a functional *lac* operator. Four independent blue colonies were chosen for further studies (plasmid numbers pHH1–pHH4). About a 40-fold increase in the uninduced level of β -galactosidase was observed for each strain.

In vitro characterisation and sequence analysis

Plasmid DNA was prepared from the transformants carrying the independently derived RI-operator plasmids. After *Eco*RI endonuclease digestion, the DNA was treated with alkaline phosphatase, extracted with phenol, and the 5' terminal labelled with ³²P. The labelled DNA was subjected to electrophoresis on a 20% polyacrylamide gel and the ³²P located by autoradiography. Two bands were observed for all four plasmids. These were excised and found to have equal radioactivity, indicating that there is one RI-operator per plasmid. The fastest migrating component was estimated to be 29 nucleotides long, the expected length of the RI-operator fragment (Fig. 2). The short RI-operator fragment was preparatively separated from the 3.5 $\times$ 10⁶ dalton plasmid DNA by filtration on Agarose A-50 followed by concentration on hydroxylapatite, or by differential elution from DEAE-cellulose (legend of Fig. 5).

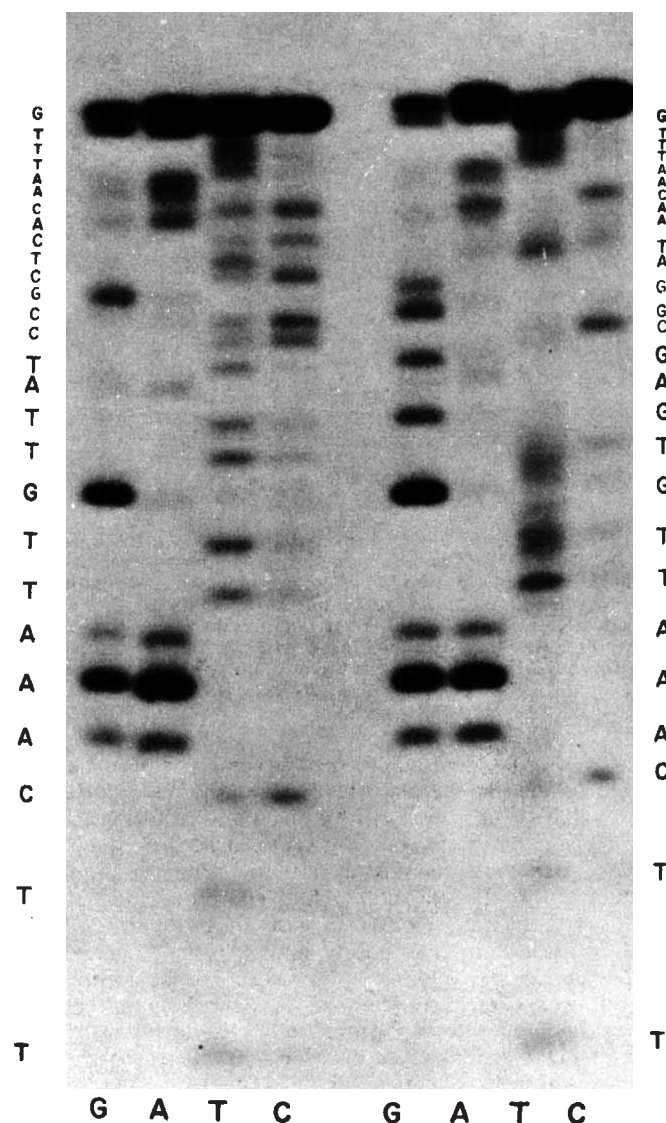
Purified RI-operator DNA from pHH2 was labelled at the 5' end, alkali denatured and the two short complementary strands separated by electrophoresis on an 8% polyacrylamide gel¹⁵. The nucleotide sequence was determined by a procedure developed by Maxam and Gilbert (personal communication). The method depends on base specific modification and cleavage of the DNA followed by separation according to length on a 20% acrylamide gel in 7 M urea. The sequence can be read from the autoradiogram shown in Fig. 3. The nucleotide sequence was identical to the 21-nucleotide sequence of the chemically synthesised *lac* operator with an AATTCA sequence at the 5' end and a TG sequence at the 3' end (see Fig. 1). The nucleotide sequences of the RI-operator DNA from all four plasmids were compared without separation of the complementary strands and no differences were evident (data not shown).

Repressor binding

The RI-operator in uncleaved plasmid DNA binds repressor very well (Fig. 4). Because supercoils in the plasmid DNA might alter the affinity of *lac* operator for *lac* repressor¹⁸, we converted the pHH2 plasmid DNA to a linear form with *Hind*III endonuclease. This enzyme cuts the pMB9 plasmid

16 h at 12 °C, dialysed against 30 mM CaCl₂, and used to transform strain RR1⁹ according to an established procedure¹⁰. The transformed culture was plated on glucose minimal medium¹⁴ containing 40 μ g ml⁻¹ of 5-bromo-4-chloro-3-indolyl- β -D-galactoside and 10 μ g ml⁻¹ of tetracycline. There were approximately 5,000 tetracycline resistant transformants per ml of culture. The reaction was followed by electrophoresis of the products in a 12% polyacrylamide gel containing 7 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA. An autoradiogram (Kodak NS X-ray film) of the gel is shown. Migration of the DNAs is from top to bottom. Lane 1: ³²P-labelled *lac* operator DNA. The slowest migrating band contains the intact operator DNA strands and the faster migrating components are presumably incomplete reactants or other contaminants. Lane 2: Ligated mixture of unlabelled *Eco*RI-linkers and ³²P-labelled *lac* operator DNA. Distinct oligonucleotide bands larger than the 21-nucleotide *lac* operator are evident and represent the operator DNA to which one, two, three or more *Eco*RI-linkers have been ligated. Lane 3: *Eco*RI digested ligation mixture. Treatment of the material shown in lane 2 with *Eco*RI restriction endonuclease reduces the large molecules to a size equal to or smaller than the RI-operator shown in lane 4. Thus the majority of the ligated material seen in lane 2 represents operator DNA bounded by *Eco*RI-linkers rather than multimers of the *lac* operator DNA. Lane 4: RI-operator DNA purified from the plasmid pHH2. The purification and ³²P-labelling of the RI-operator are described in the legend to Fig. 3.

Fig. 3 DNA sequence analysis of the RI-operator fragment. Purified pHH2 plasmid DNA was treated with *EcoRI* endonuclease to release the small operator fragment. The cleaved DNA was dephosphorylated by treatment with alkaline phosphatase (1 unit per 200 pmol of 5' termini in 25 mM Tris-HCl, pH 8.0, 1 mM EDTA), followed by phenol extraction and G-50 Sephadex gel filtration. The dephosphorylated DNA was ^{32}P -labelled as described in the legend of Fig. 2. The small RI-operator DNA was separated from the 3.5 megadalton linear plasmid DNA by chromatography on DEAE-cellulose. The RI-operator DNA was eluted with 0.8 M triethylammonium carbonate at pH 8.0 and concentrated by lyophilisation. The complementary strands of the RI-operator were separated by denaturation in 0.1 M NaOH followed by electrophoresis on an 8% polyacrylamide gel in 50 mM Tris-borate pH 8.3, and 1 mM EDTA¹⁵. The two strands separated completely and, after elution from the gel, were used for sequence analysis. The sequence determinations were carried out according to a protocol kindly supplied by Maxam and Gilbert (to be published). The procedure depends on base specific cleavage of 5'-labelled DNA, followed by separation of the fragments according to size by electrophoresis on 20% acrylamide gels under denaturing conditions (7 M urea). Preferential cleavage at guanosine residues was achieved by methylation with dimethylsulphate, depurination by heating to 90 °C at pH 7.0, followed by alkali treatment. Cleavage at adenosine residues was achieved by depurinating the methylated DNA at 0 °C for 1 h in 0.1 N HCl. Preferential modification and subsequent cleavage at cytidine residues was obtained by treatment with hydrazine in the presence of 1 M NaCl. Cleavage at thymidine residues results when hydrazine treatment is carried out in the absence of NaCl. The darkest band at each fragment size indicates which condition caused preferential cleavage, and thus identifies the base at which cleavage occurred to give the fragment. In practice, each cleavage condition is only partially specific, but the cleavage pattern for all four conditions run in parallel allows the sequence to be determined, especially when both strands are analysed. The right four lanes of the autoradiogram shows the cleavage pattern for the upper strand. The left four lanes show the lower strand. For each set the G-specific cleavage is on the left followed by A-specific, T-specific, and C-specific. The first two nucleotides at the 5' end of each strand were not determined by this method but are known from the specificity of *EcoRI* endonuclease.



about 200 base pairs from where the RI-operator is located⁹. Linear pHH2 DNA competes even more effectively for repressor; only a threefold molar excess gives 50% competition with ^3H - λ plac DNA. This result strongly suggests that when the RI-operator is sequestered in high molecular weight DNA, it has an affinity for repressor close to that of natural *lac* operator.

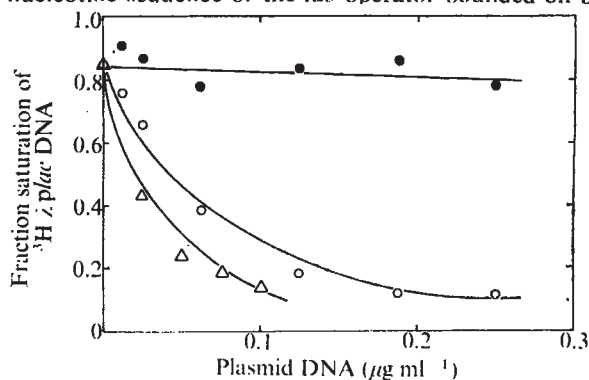
Direct filter binding experiments with purified ^{32}P -labelled RI-operator DNA fragments were also done and they demonstrate clearly that the small RI-operator DNA fragment binds *lac* repressor (Fig. 5). The filter retention is eliminated by isopropylthiogalactoside (IPTG), an inducer of the *lac* operon. The plateau of filter retention is only 20% for the RI-operator DNA, whereas the filter retention

of λ plac DNA is usually about 50%. The lower retention efficiency for the RI-operator DNA probably is a reflection of the lower binding affinity of the small fragment for repressor. Our preliminary competition data indicate that the excised RI-operator fragment has at least a 100-fold reduced affinity for repressor, compared with intact pHH2 plasmid DNA.

Chemically synthesised DNA and cloning

In this paper and the paper by Mariani *et al.*¹⁷, are reported the first successful construction of plasmids carrying a functional genetic element derived from chemically synthesised DNA. The plasmids described here carry the 21-nucleotide sequence of the *lac* operator bounded on both

Fig. 4 Competition for *lac* repressor by plasmid DNA. Various amounts of unlabelled plasmid DNA were mixed with 0.025 μg of ^3H - λ plac DNA and then an equimolar amount of *lac* repressor was added. This amount of repressor was found to give 85% saturation of the operator in ^3H - λ plac DNA in the absence of competing DNA. The reaction volume was 0.4 ml and the buffer was 10 mM KCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% (v/v) dimethylsulphoxide, and 50 $\mu\text{g ml}^{-1}$ of bovine serum albumin. After 30 min incubation at room temperature, 0.15-ml aliquots were filtered in duplicate through nitrocellulose filters according to the procedure of Riggs *et al.*²⁰, and washed once with 0.2 ml of buffer without bovine serum albumin or dithiothreitol. ●, pMB9 plasmid DNA; ○, pHH2 plasmid DNA; △, *HindIII* endonuclease-treated pHH2 plasmid DNA.



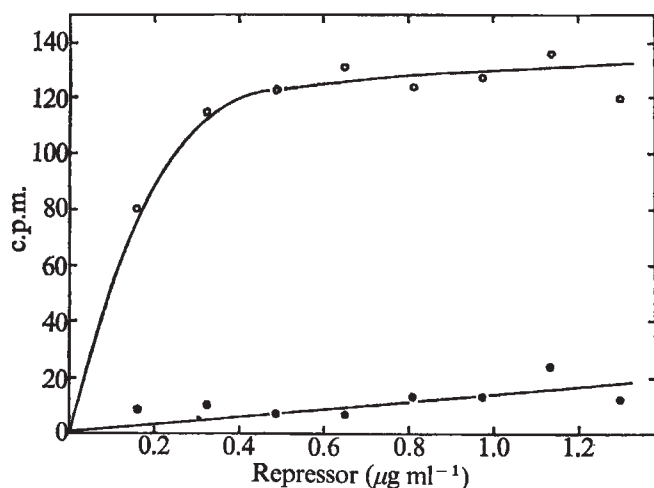


Fig. 5 Binding of *lac* repressor to purified ^{32}P -RI-operator DNA. The purified small RI-operator fragment was labelled with ^{32}P -phosphate by T4 polynucleotide kinase treatment. Varying amounts of *lac* repressor were added to 2.6 ng of purified RI-operator DNA. The reaction volume was 0.5 ml and the buffer was that used in Fig. 4. After 20 min at room temperature, 0.2 ml aliquots were filtered and washed as in Fig. 4. ●, No IPTG; ○, 10^{-3} M IPTG.

sides by *EcoRI* endonuclease restriction sites. The *lac* operator cloned in the plasmids functions *in vivo* by binding the available *lac* repressor molecules, resulting in the constitutive synthesis of β -galactosidase. *EcoRI* endonuclease can be used to excise the small RI-operator fragment from the plasmid DNA. Therefore, this plasmid provides a continuous source of a small *lac* operator DNA, which will greatly facilitate many physicochemical and other experiments. We are now trying to construct plasmids containing several RI-operators polymerised in tandem. Such a polyoperator plasmid will provide enough *lac* operator DNA for crystallisation of repressor-operator complexes and the structural analysis of the mechanism of a sequence-specific protein-DNA interaction.

These studies show that the 21-nucleotide sequence of the "central" *lac* operator provides for almost all of the specific *in vitro* interactions with *lac* repressor. As shown in Fig. 1, Dickson *et al.*³ noted additional regions of symmetry on each side of the central 21-base-pair operator. This observation led to speculation that the repressor might interact specifically with a larger DNA sequence (35 base pairs). We

find that the RI-operator sequence, when it is sequestered in large molecular weight DNA, has an affinity for repressor within a factor of three of that of natural *lac* operator in λ plac DNA. Therefore, the symmetrical sequences in the "extended operator" are not obviously involved in this protein-DNA interaction. When RI-operator is cut from the plasmid DNA, the affinity for repressor is lowered greatly. Therefore, it seems necessary to have DNA on both sides of the 21-base-pair operator for "normal" interactions, but the sequence of this DNA is probably not critical.

The method used to generate the RI-operator fragment, that of the blunt end joining of a short restriction enzyme substrate (linker) to each end of the DNA of interest, is generally useful for DNA manipulation and cloning. For example, *EcoRI* "linkers" or other endonuclease substrate linkers now can be added to any blunt ended DNA molecule. The combination of chemical synthesis technology with cloning technology will provide a powerful approach to many problems, including the chemical synthesis of long DNA sequences.

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letters to nature

The hard X-ray spectrum of Cyg X-1 during the transition in November 1975

We wish to report an observation of the hard X-ray spectrum of Cyg X-1 during a transition to the high state in November 1975. The measurement was obtained with a balloon-borne X-ray detector in the 25-150 keV range. This third upward transition of Cyg X-1 in 1975 was also detected in the few-keV energy range by the Ariel V all-sky monitor (S. S. Holt, L. J. Kaluzienski, E. A. Boldt, and P. J. Serlemitsos, unpublished). Our high energy data seem to confirm the characteristic spectral time variation of Cyg X-1: a soft X-ray intensity

increase is correlated with a decrease of the hard X-ray intensity. The similarity of Cyg X-1 and the transient source A0620-00 with respect to such spectral variation has been pointed out by Coe *et al.*¹

Cygnus X-1 was observed on November 4, 1975 from 2245 until 2330. The apparatus was launched from Palestine, Texas and floated for 5.5 h at an altitude corresponding to a pressure of 4.3 g cm⁻². The data were obtained with an array of six 100-cm² NaI (Tl) detectors which are part of an X-ray burst experiment under construction. The counts of each detector were sampled in four energy channels for equal periods of time. The data handling as well as the film recording were designed

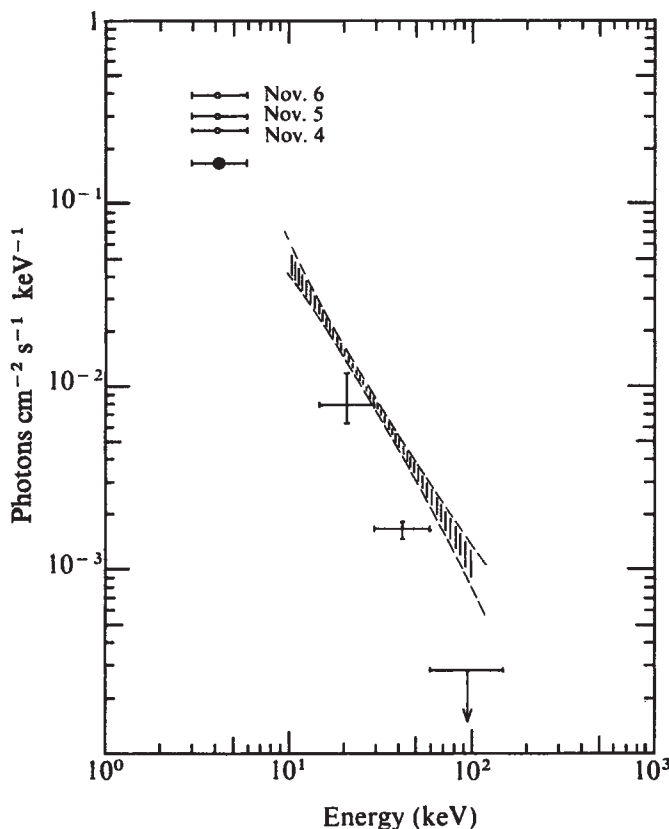


Fig. 1 Spectra of the X-ray source Cyg X-1. The black dot and hatched area indicate average spectra of Cyg X-1 in the 'normal state'. $\circ$, Daily averages of the intensity in the 3-6 keV band for three successive days during the upward transition of Cyg X-1 (from ref. 4). The crosses are our data.

to suit the apparatus on future transatlantic balloon flights. In this short test flight we used a $4 \times 22''$ passive collimator to separate X-ray sources. The gondola was orientated by an azimuth control system. The background flux was observed shortly before and after the Cyg X-1 exposure. In the 30-60-keV range, 7×10^{-3} counts $\text{cm}^{-2} \text{s}^{-1} \text{keV}^{-1}$ were found. After subtracting the background we fitted a power spectrum to the data taking into account absorption, collimator response, energy resolution and the probability of escape of an iodine K photon. All detectors were calibrated by measuring the 60-keV line of a ^{241}Am radioactive source and the iodine escape peak at ~ 30 keV.

The resulting X-ray intensity of Cyg X-1 is shown in Fig. 1. For comparison we plotted typical normal state spectra of Cyg X-1, which are remarkably constant as often observed. The soft X-ray intensity data (from ref. 4) are daily averages for November 4, 6 and 5, 1975. Our data combined with the soft X-ray measurements suggest a single power law spectrum from 3 to 80 keV with an increasing spectral index during the upward transition of Cyg X-1 to the high state.

A power spectrum is expected if one assumes the inverse Compton effect as the basic mechanism which produces the hard X-ray tail of Cyg X-1 (ref. 2). These models require a very luminous source of soft photons inside an optically thin, hot ($kT_e \approx 100$ keV) disk. Spectral time variation may be caused by a varying intensity of the inner soft photon source and a stable hot cloud. Spherical accretion on to a black hole has been suggested (ref. 3 and U. Anzer, G. Börner, and P. Mészáros, unpublished). Temperatures of $> 10^9$ K may be produced by the dissipative heating of the infalling gas. The X-ray spectra could then arise from bremsstrahlung which would also explain the spectra.

We thank the staff of the NCAR-balloon base for their experienced help in launching our balloon.

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Ball lightning as electromagnetic energy

NONE of the theories¹⁻⁸ proposed to explain ball lightning seems able to account satisfactorily for the experimental evidence⁹⁻¹⁴. Jennison¹⁵ has suggested that ball lightning is a spherical stable standing wave of electromagnetic radiation. The proposal that ball lightning in effect consists of electromagnetic field energy trapped in an evacuated cavity of approximately spherical shape, with an ionised sheath separating it from the atmosphere, merits further examination.

The energy density inside the ball will be magnetic ($B^2/2\mu_0$), electric ($\epsilon_0 E^2/2$) or both. For equilibrium, the ball must exert an average pressure at all points on its surface equal to atmospheric pressure. The standard boundary conditions for a microwave cavity with conducting walls are **E** normal and **B** tangential to the boundary. For these conditions the outward electromagnetic pressure is $[(B^2/2\mu_0) - (\epsilon_0 E^2/2)]$. Now the largest estimate^{1,8} of the energy density in a ball ($\sim 4 \times 10^9 \text{ J m}^{-3}$) is 4×10^4 times the atmospheric pressure expressed as an energy density. The average magnetic energy density at all points on the surface of the ball must therefore be very nearly equal to, but slightly greater than, the average electric energy density. This is the main difficulty, but further problems include stability, power loss and mechanism of formation of the ball.

A mechanism consistent with recently reported observations¹⁶, for the formation of a simplified 'cylindrical' ball is depicted in Fig. 1. A cloud of positive space charge is attracted towards a negative leader stroke as it approaches the ground (Fig. 1a). There is some misalignment, however, which causes parts of the two regions of space charge to spiral around each other (Fig. 1b). The end result is an electric dipole configuration rotating in the atmosphere just above the ground (Fig. 1c). This departure from the normal cloud to ground discharge mechanism requires an appropriate asymmetry in the geometry (for example, a small building or a tree⁹ in the immediate vicinity). It is therefore a comparatively rare event, as required by the observed incidence of ball lightning¹⁶. It is well known from data on lightning return strokes¹, that once the atmosphere has been ionised, the speed of a lightning stroke can exceed 10^8 m s^{-1} . It is an important feature of this model that the peripheral speed of the final dipolar rotating field pattern should be $> c$, the velocity of light. Given that the space charge regions are not reduced to charges of one sign only, this is quite possible, since there is then no requirement for individual charged particle velocities to be $> c$, (phase velocities $> c$ are commonplace in wave-guides and in pulsar rotating fields¹⁷, both of which have features in common with this model).

A sketch of a rotating electric dipolar field pattern in a long cylindrical cavity is shown in Fig. 2. The return path for the current I in the wall is by way of the displacement current in the cavity which may be written as $(\nabla B \times \hat{Z})/\mu_0$. The vacuum field inside the cavity may be obtained by solving the equations

$$\nabla^2 B - (\omega^2/c^2)\partial^2 B/\partial t^2 = 0$$

$$(\nabla B \times \hat{Z}) + (\omega/c^2)[\hat{r}\partial E_r/\partial\theta + \hat{\theta}\partial E_\theta/\partial\theta] = 0$$

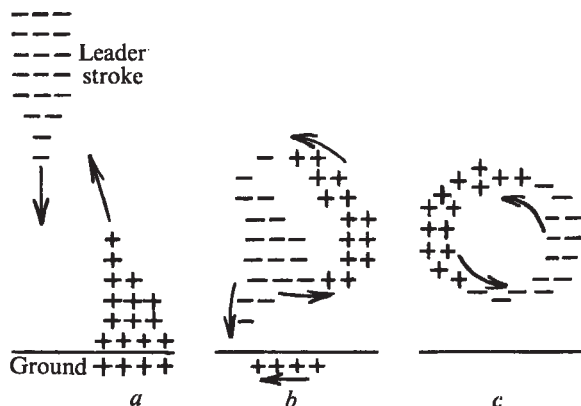


Fig. 1 Successive stages (a-c) in the suggested mechanism of ball lightning formation.

subject to the condition that E is normal to the boundary. The r -component of the second equation gives, for the rotating dipolar field considered

$$B = -(r\omega/c^2)E_r$$

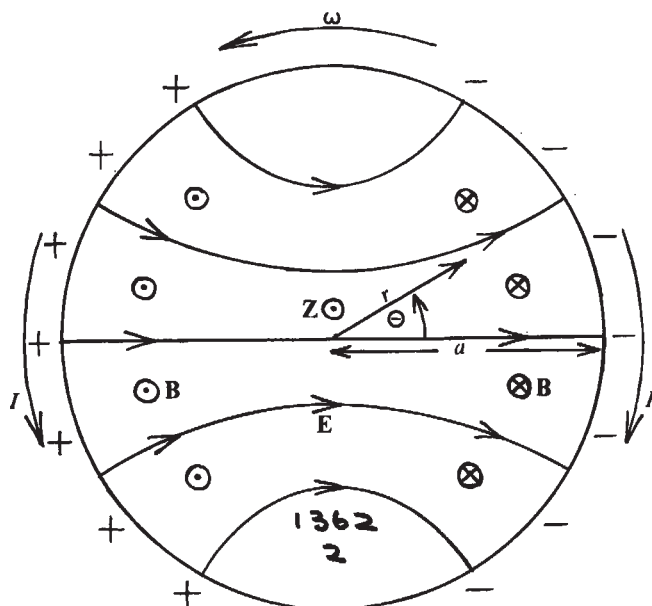
Thus at the boundary (radius a), where E_θ is zero, the net outward electromagnetic pressure is

$$(B^2/2\mu_0) - (\epsilon_0 E^2/2) = (B^2/2\mu_0)[1 - (c^2/a^2\omega^2)]$$

Therefore the peripheral velocity ($a\omega$) has to be $> c$ so that the net electromagnetic pressure be directed outwards. The electromagnetic pressure does of course vary with rotation angle, but the frequency is too high for the atmosphere to respond appreciably to the variations. The ratio of the electromagnetic energy density to the electromagnetic pressure at the sheath and hence to the atmospheric pressure, can be made as large as is required to fit the observations. The particles in the sheath will move at the convective velocity ($E \times B/B^2$) with a small counterstreaming velocity superimposed. In the frame moving at the convective velocity the radial electric field is zero and a small outward ($J \times B$) force balances the atmospheric pressure. Compared with the radial component of electric field in the rest frame, the tangential electric field in the sheath associated with the small counterstreaming (conduction) current will be negligible. The ball does not discharge internally because the radial electric field seen by the convected sheath is zero.

For an energy density in the ball of $4 \times 10^8 \text{ J m}^{-3}$, the asso-

Fig. 2 Schematic diagram of a rotating electric dipolar field pattern in a long cylindrical cavity with conducting wall. No attempt has been made to show retardation of the field.



ciated values of E and B are $\sim 2 \times 10^{10} \text{ V m}^{-1}$, and 70 T respectively. For a cylinder of $\sim 10 \text{ cm}$ diameter⁹ and 30 cm length, the total energy is $\sim 10^7 \text{ J}$, that is, 1–10% of the energy available in a typical lightning discharge; and the charge is 0.02 C , which is $\sim 10\%$ of a typical leader charge⁴, suggesting that only a fraction of the leader charge goes into formation of the ball, the remainder discharging to ground in the normal way (Fig. 1b).

In principle there is no difficulty in extending this model to the spherical case. To avoid nodes of zero electromagnetic pressure on the rotation axis, additional precessional velocities need to be introduced. Any air penetrating the surface of the ball near the rotation axis will be rapidly ionised and expelled. The forces required are likely to be provided by a secondary flow of conduction and convection currents across the rotation axis, so that the ball effectively generates its own precessional mechanism. The average electromagnetic pressure can then be made the same at all points on the surface, as required for equilibrium. This field computation would, however, be very complicated. Nevertheless, the most stable configuration is the most likely to occur in practice, and is likely to be approximately spherical, although any rapidly precessing volume of revolution would be indistinguishable from a sphere by the naked eye. The exact shape to be expected would depend on detailed energy considerations. The MHD instabilities that occur in plasma confinement experiments are not likely to occur with this model, because the field is inside rather than outside so that the boundary curvature is everywhere favourable, and also because there will undoubtedly be a high degree of shear in the boundary magnetic field. The wall current is likely to be largely convective, with only small conduction currents needed to keep charge velocities $< c$. Power loss through ohmic heating of the sheath is therefore expected to be small.

There is also likely to be a small leakage of the field through the sheath, which could be sufficient to maintain the ball at a steady distance from surrounding structures, but insufficient to cause motion in small metal objects nearby¹⁵. On the other hand, strong interaction with metal objects penetrating the surface of the ball would be expected¹⁴. The energy in the ball could either be entirely dissipated by ohmic heating of the sheath and radiation through it, in which case the ball would disappear quietly, or it could be dispersed by a perturbation leading to an instability, in which case the ball would disintegrate explosively. Both explosive and non-explosive terminations have been observed⁴. There is no reason, according to this model, why ball lightning should not drift into buildings¹¹, or be found in aeroplanes¹⁰, since it transports its own energy. In the latter case, the ball would either have to pass through a glass window, or be formed inside the aeroplane. If in fact lightning were to strike an aeroplane near one of its windows, it is quite possible that sufficient electric field and ionisation would be set up inside the aircraft for ball formation to occur as described here. The metal surrounding the window would take the place of the ground, and the geometry would favour the necessary spiralling motion.

Undoubtedly this model will require modification as further characteristics of ball lightning become established. It does suggest, however, that the electromagnetic field inside the ball is likely to be the energy source rather than nuclear reactions¹, antimatter⁷, continuous energy input from an external field^{5,6}, or unusual forms of gas discharge energy^{2,3}.

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Radiocarbon chronology of late Quaternary lakes in the Arabian Desert

A GOOD deal of information on late Quaternary moist periods in East Africa and Australia, derived largely from the dating and analysis of lake sediments, has been collected over recent years^{1–4}. Although fossil lake beds have long been known to exist in Arabia^{5–7}, they have received scant attention. I report here the results of a preliminary attempt to establish the age of a series of late Quaternary lake sediments in the Rub' al Khali desert of the Arabian Peninsula. More detailed palaeoclimatic implications will be considered elsewhere later.

The Arabian Peninsula (Fig. 1) is essentially a plateau which dips gently towards the north-east and can be subdivided into a western (Precambrian) igneous shield and an eastern province of sedimentary rocks. Alluvial sands, gravels and silts obscure a dendritic drainage system which was established in Quaternary times^{7–9}. These sediments are in turn partially blanketed by wind-laid sands, especially in the eastern basins. The largest of these basins is the now extremely arid Rub' al Khali.

Lacustrine deposits are intimately associated with the aeolian and alluvial deposits and, though insignificant in dimensions, hold the key to the late Quaternary geomorphological and climatic evolution of the area. The deposits consist of calcareous and often fossiliferous marls, clays and silts. They characteristically form flat-topped benches and mounds, often on the lee of longitudinal dunes and in the 'corridors' between the dunes where recent deflation has removed the superficial deposits. Stratigraphical and morphological criteria indicate the presence of two main generations of lake beds, the older resting on alluvium, and the younger on aeolian sands which antedate the modern (red) dune sands. The lake beds seem to reflect centripetal runoff into deflated hollows.

Fossil Lake Mundafan (Fig. 1), one basin in which both sets of deposits are represented, displays 24 m of lake beds

Table 1 Radiocarbon dates from sites mentioned in text

Field no.*	Laboratory no.	Material	Age (yr b.p.)
Series A			
MF 1	UGa-1218	Marl	17,460 ± 245
MF 2	UGa-1203	Marl	21,090 ± 420
MF 3	UGa-1217	Marl	21,280 ± 275
Site 1	I-6987	Marl	21,400 ± 450
MF 4	UGa-1202	Marl	22,345 ± 415
MF 5	UGa-1211	Marl	22,965 ± 390
MF 6	UGa-1209	Marl	23,075 ± 425
MF 7	UGa-1213	Marl	24,145 ± 400
MF 8	I-7427	Algal limestone	25,660 ± 810
Site 2	I-7447	Marl	27,160 ± 940
MF 9	UGa-1220	Marl	28,750 ± 615
MF 10	UGa-1219	Shells	29,595 ± 780
Site 3	I-6988	Shells	29,660 ± 1,400
MF 11	I-7111	Shells	36,300 ± 2,400
Series B			
MF 12	UGa-1216	Algal encrustation	6,100 ± 70
Site 4	I-6410	Shells	6,520 ± 115
MF 13	UGa-1207	Diatomaceous marl	7,040 ± 115
Site 5	I-7307	Shells	7,160 ± 115
MF 14	UGa-1204	Marl	7,190 ± 85
MF 15	UGa-1206	Diatomaceous marl	7,265 ± 80
MF 16	UGa-1208	Shells	7,400 ± 210
MF 17	UGa-1205	Diatomaceous marl	7,770 ± 90
MF 18	UGa-1212	Marl	8,060 ± 95
MF 19	UGa-1221	Marl	8,155 ± 85
MF 20	UGa-1222	Shells	8,565 ± 110
MF 21	UGa-1214	Charcoal/ash	8,800 ± 90
Series C			
MF 22	UGa-1215	Calcareous siltstone	11,465 ± 115
MF 23	UGa-1210	Marl	14,965 ± 195

*MF refers to Lake Mundafan samples. Sites of other samples are indicated in Fig. 1.

interstratified with alluvial and aeolian sands. The principal sources of runoff were probably the backslope of the eastward-dipping Wajid plateau and the foreslope of the eastward-dipping Al Arid (Jabal Tuwaiq) escarpment. The lake beds of the Mundafan basin are thought to extend beneath the alluvium and aeolian sands 150 km northward along the Al Arid escarpment.

Radiocarbon dating of lake beds from the Mundafan basin and from five other smaller depressions further inside the Rub' al Khali (sites 1–5 in Fig. 1) supports the contention that two main periods of high water level occurred in the area during the late Quaternary. The dates (Table 1) place the first period (series A) between ~36,000 and 17,000 yr b.p., with a concentration of dates between 21,000 and 30,000 yr b.p., and the second period (series B) between ~9,000 and 6,000 yr b.p. A third period of minimal and low-lying lake bed deposition (series C) separates the two.

At Lake Mundafan, extensive wind erosion, local slumping, possible changes in basin configuration, and the fluctuating nature of the various water levels combine to complicate stratigraphic relationships of the beds from which the dated material derives. Details remain to be worked out. Generally, beds of Series A occupy high parts of the basin and beds of series B and series C occupy low parts.

The dates reported here are in close agreement with dates reported from East Africa and Australia^{1–4}. Additional dates and further analysis of Arabian lake bed material should help define more precisely the late Quaternary palaeoclimatic sequence of the area.

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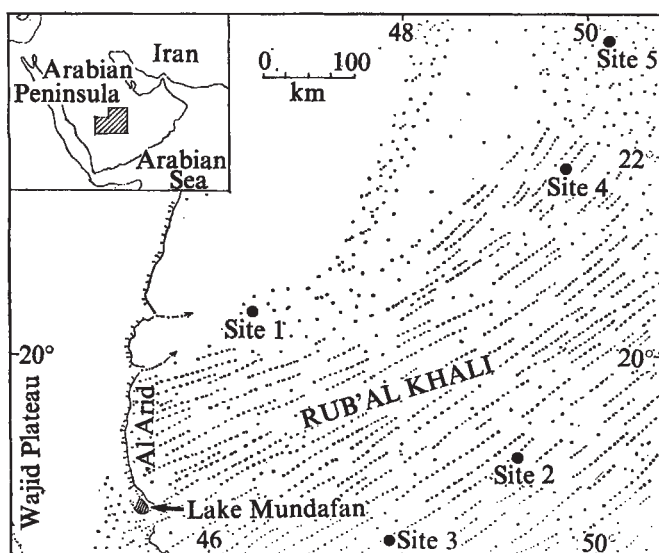
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Fig. 1 Location map of places mentioned in text.



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Effect of magnetic field on reduction of nickel oxide

"In 1872, US patent No. 133099 on the use of a magnetic coil for 'Improvement in Reduction of Ores, etc.' was granted to Abraham T. Hay, of Burlington, Iowa¹. It was a failure." Thus stated Skorski² who a century later reported that the reduction rate of haematite (Fe_2O_3) with H_2 was considerably enhanced by application of 500- and 1,400-oersted magnetic fields as compared with reduction in the Earth's field (~ 0.5 oersted). Skorski² also used CH_4 and CO for the reduction, but found the rate in these cases was slower in a strong magnetic field than in the Earth's field. He concluded that the effect must be dependent on the magnetic properties of H_2 itself rather than on those of Fe and its oxides. This notion was immediately challenged by Svare³ who demonstrated that the magnetic properties of H_2 are not significantly different from those of CO and CH_4 in the context of the reaction involved. Peters⁴, however, argued that the observed acceleration in reaction rate is predictable from thermodynamics theory when product and reactant species differ widely in their magnetic properties—as do Fe_2O_3 and metallic Fe (or NiO and metallic Ni) for example.

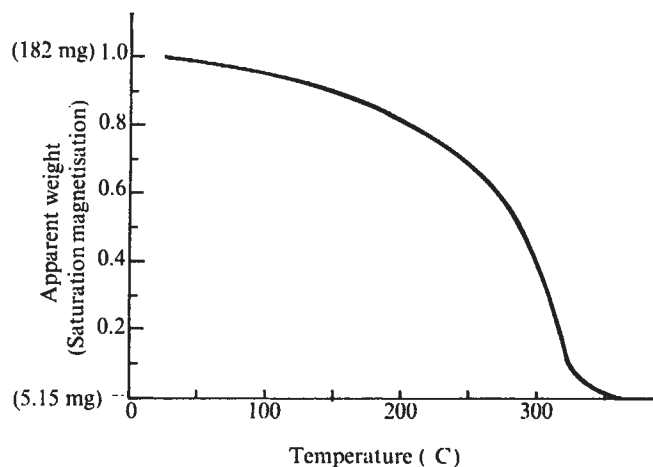
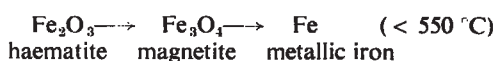


Fig. 1 Variation of the saturation magnetisation of Ni metal with temperature, as recorded by the electrobalance used in this work. Note that the apparent weight (saturation magnetisation) of Ni is much greater than its actual weight.

One can readily view this argument qualitatively in terms of Le Chatelier's principle.

We recently began a systematic study of the effect of a strong magnetic field ($\sim 4,200$ oersted) on oxidation-reduction systems involving strongly magnetic products of reactants. The Fe-iron oxide system is, however, relatively complicated



Thus Skorski² examined the above reduction on an 'observation after the fact' basis—the final product probably being an unknown mixture of Fe_2O_3 , Fe_3O_4 and Fe. The interpretation of results is thus considerably complicated.

We present here results using a sensitive technique whereby observations are continuously recorded on a real-time basis during constant temperature reduction of NiO to Ni. Since Ni is ferromagnetic whereas NiO is paramagnetic, Peters⁴ criterion

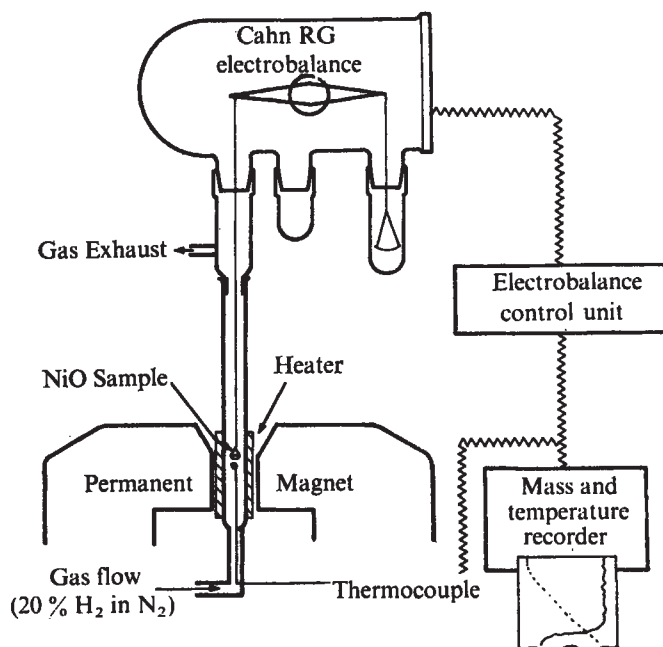
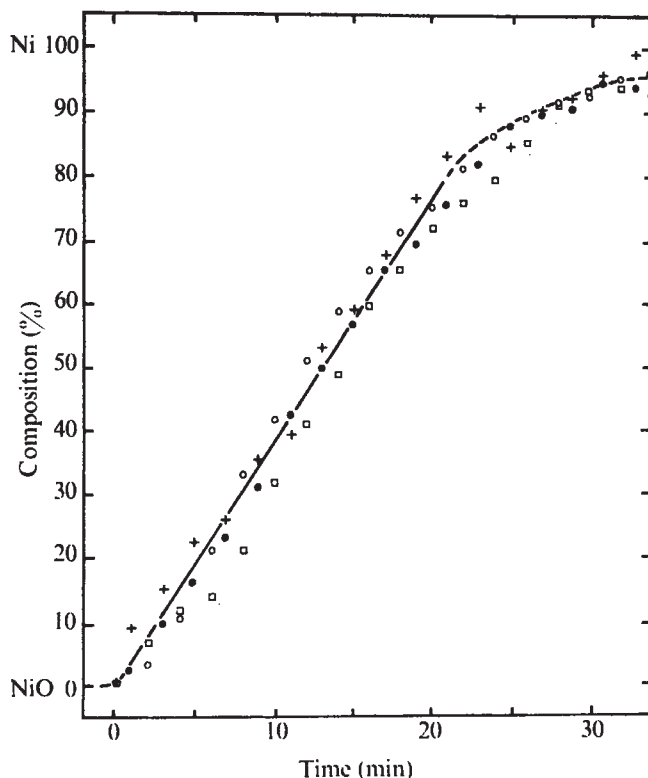


Fig. 2 Schematic diagram of the Cahn electrobalance used in this work. The system continuously records the weight of the sample as the reaction proceeds.

that product and reactant magnetic properties differ widely is met. We thus incorporated two distinctive features into our work: first, we selected a simple metal-metal oxide system; only NiO and Ni are involved. Only NiO occurs in the ordinary chemistry of nickel. Higher oxidation states of nickel are quite

Fig. 3 Reduction of NiO to metallic Ni with 65 ml min^{-1} flow of 20% H_2 in N_2 carrier at 295°C . This experiment was conducted in the Earth's magnetic field. Points were hand-read from a strip chart recording for construction of Figs 3 and 4 so they could be directly compared. The mass data were recorded continuously: $\square$, 2.90 mg NiO ; $+$, 3.08 mg NiO ; $\bullet$, 0.92 mg NiO ; and $\circ$, 1.14 mg NiO . The solid bold line through the data points is a least squares best fit straight line fitted to the points from 0 to 20 min. It is for comparison only.



unstable; second, we continuously monitored the course of the reaction.

The behaviour of Ni in the presence of a large magnetic field ($\sim 4,200$ oersted) is well known, as is the temperature dependence of this behaviour (illustrated in Fig. 1 for our system). This behaviour was determined by suspending a small sample from a sensitive balance (see Fig. 2). The application of a strong inhomogeneous magnetic field to the sample results in a force of attraction—measured as an apparent weight which is many times greater (~ 40 times for Ni) than the actual weight of the sample. The apparent weight (saturation magnetisation) decreases with increasing temperature until it essentially vanishes at the Curie temperature. Heating and cooling curves were indistinguishable.

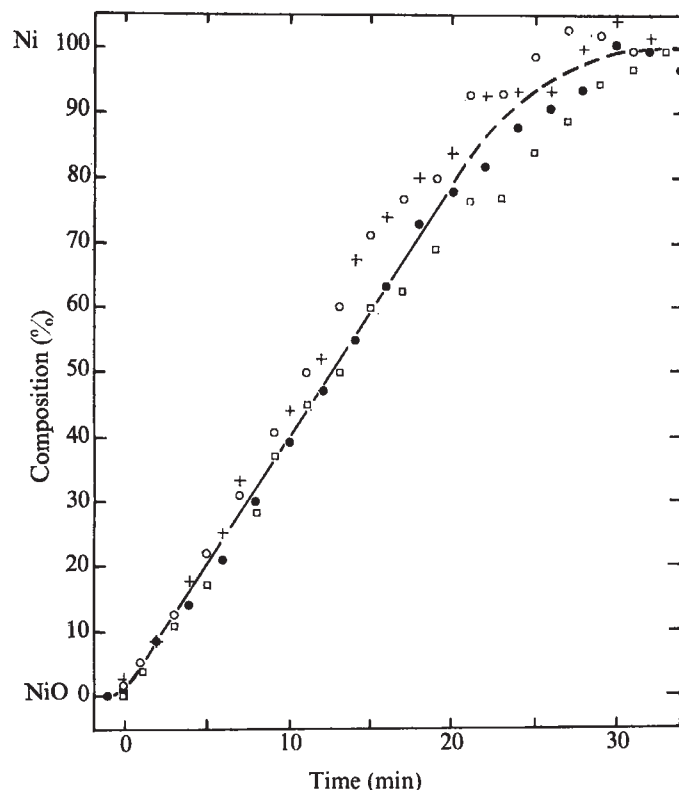


Fig. 4 Reduction of NiO to metallic Ni. Experimental conditions were the same as those in Fig. 3, except that a strong magnetic field ($\sim 4,200$ oersted) was applied here. $\square$, 2.58 mg NiO; $+$, 1.29 mg NiO; $\bullet$, 2.08 mg NiO; and $\circ$, 2.87 mg NiO. The line is again a least squares best fit to the data exhibited in Fig. 4 in the early portion (0–20 min).

To monitor the change in NiO and Ni during the course of the reduction of NiO without application of a magnetic field, 20% H_2 in N_2 carrier was passed through the balance system (Fig. 2 with the magnet removed) for ~ 1 h to thoroughly flush the system with 1–3 mg samples of NiO in place. The temperature was then quickly raised to $295^\circ C$ (~ 10 min) and the weight of the sample was continuously recorded as a function of time. The observed weight change directly reflects the relative amounts of Ni and NiO in the sample at any time. The early portion of the curve in Fig. 3 (~ 0 –20 min) was approximately linear and the bold central line shows a least squares best fit to the average of the four runs. Each of these runs were conducted in the Earth's magnetic field (~ 0.5 oersted).

To ascertain the change in this reduction rate (if any) caused by the application of a strong magnetic field, we used the same experimental system (Fig. 2) but with the $\sim 4,200$ oersted magnet in place to influence the sample during the course of the reduction. In this case the apparent weight change was greatly exaggerated during the course of the reduction as the non-magnetic (paramagnetic) NiO was converted into ferro-magnetic Ni, which under the influence of the magnetic field

yielded an apparent weight many times the actual weight of the sample (Fig. 1). Again four independent reductions, this time carried out under a strong magnetic field, were conducted and the results are shown in Fig. 4. Again a least squares line was fitted to the approximately linear portion of the curve. The difference in slope between the line in Figs 3 and 4 was only 1.3%, well within our experimental uncertainty. It is apparent from an examination of Figs 3 and 4 that any difference in the reduction rate of NiO, with or without the application of a magnetic field, is quite small and is not discernible by our technique.

We now intend to investigate the more complex iron-iron oxide system maintaining control over reaction products by carefully mixing reaction gases so as to produce an oxygen fugacity in which the desired product is stable whereas the others are not⁵.

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Charge transport by solid particles in liquid dielectrics

ONE of the more controversial aspects of the study of liquid dielectrics has been the role of natural particles in the mechanism of charge transport (see, for example, the recent review by Gallagher¹ and references therein). Theoretical estimates of the charge carried by individual particles are discrepant by several orders of magnitude. However well purified and filtered a liquid sample may be, as soon as it is subjected to electrical stress such particles may be seen in motion between the electrodes by observing scattered light from them through a microscope. They are of indeterminate composition and $\sim 1 \mu m$ in diameter. In the first attempt to make a direct study of charge transport by natural particles² reliance had to be placed on a correlation involving the human senses, which was valid for low voltages and was assumed to be capable of extrapolation to higher voltages, where speeds are too great for such methods to be used. Also, since the particles exhibit a dwell-time on the electrodes which is very much greater than the transit time the experimental problem of capturing the charge waveform is considerable. Here we report preliminary results of an attempt to correlate the waveforms formerly attributed to solid particles with physical evidence of actual particle motion obtained by observation of the light scattered from them.

As a problem in measurement this is one of intriguing complexity. It is necessary to capture two fast waveforms, a charge signal of the order of femto coulombs and a light signal of the order of a tenth of a lumen. These signals occur at random and are corrupted by noise. Indeed, the first attempt to be made to record the motion of a particle by means of scattered light failed. This involved focusing the image of the centre of the gap on to the cathode of a photomultiplier, the image of the electrode surfaces being masked off. Unfortunately, the

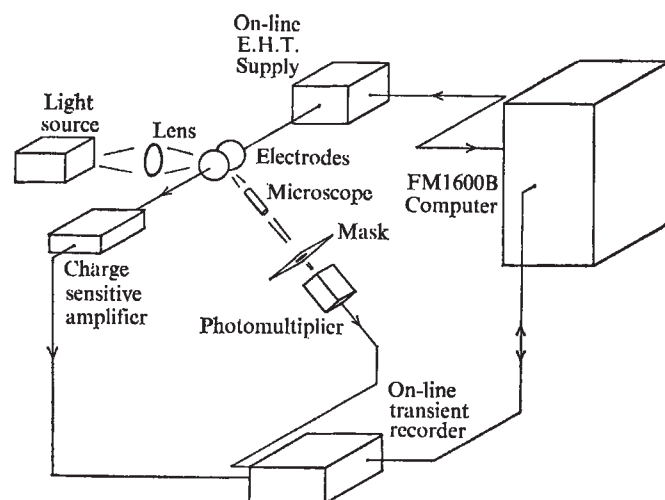


Fig. 1 Experimental arrangement for the simultaneous monitoring of motion and charge transport by natural particles. The 1-cm diameter spheres are 100 μm apart, and light scattered through 50° is collected by the microscope. The mask at the real image selects one electrode surface for transmission to the photocathode. The light and charge waveforms are captured by the on-line transient recorder and transmitted to the computer.

combination of high speed and low level of scattered light produced a signal which was irretrievable from noise. It was found, however, that if the image of one electrode surface was projected on to the photocathode, the arrival and departure of particles could be recorded as step waveforms in the light scattered at the electrode.

Fig. 2 Captured waveforms from the on-line incremental plotter. *a*, A typical step produced by a particle leaving the electrode. There is a slight delay from noise filtering. *b*, The corresponding charge waveform. The decay following the step rise is introduced artificially. *c*, The result of averaging 36 charge waveforms. These were adjusted horizontally to maximise registration of the corresponding light waveform steps.

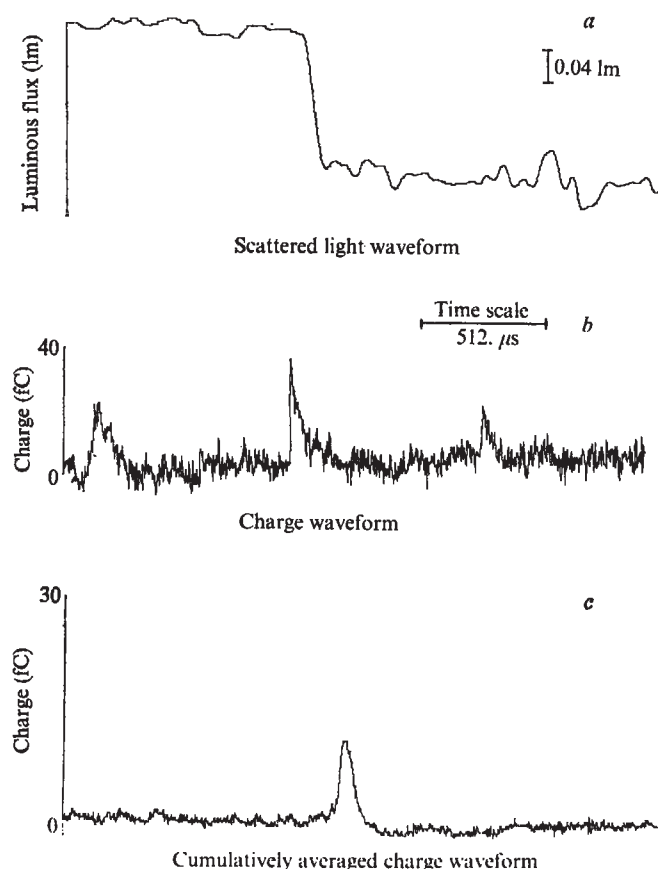


Figure 1 shows the experimental arrangement in diagrammatic form. The photomultiplier is placed at an angle of 50° to the axis of incident light and the mask is set so that scattered light from only one of the pair of spherical electrodes is recorded. Meanwhile a charge-sensitive amplifier is used to monitor the increments of charge at the cathode (though as is the practice with nuclear detectors, an artificial decay is provided in the response to prevent the 'buildup' which would saturate the amplifier). The residual problem of recording and correlating these randomly occurring waveforms was until recently a formidable one, but the introduction of the on-line transient recorder³⁻⁵ has made it quite simple.

Figure 2*a* and *b* shows scattered light and charge waveforms recorded simultaneously by the transient recorder and plotted by the on-line computer. It will be seen that there is a coincidence suggesting that the characteristic charge transient is indeed the product of charge conveyance by a solid particle. Single instances, however, do not establish a definite correlation, and to do this the following experiment was conducted.

Pairs of waveforms like those in Fig. 2*a* and *b* were acquired repetitively at random by the on-line transient recorder. Owing to the effects of noise in the light waveforms, which are used to trigger the transient recorder, slight misalignment can occur between one light waveform and another. Therefore, within the computer the waveform pairs were shifted slightly to give best registration of the light waveforms only. Meanwhile the corresponding charge waveforms were cumulatively averaged. The sharp artificial decay is advantageous in this application since it yields a short pulse which is more difficult to register and therefore is a more demanding test for correlation. The results of 36 such repetitions are shown in Fig. 2*c*. While the shape of the charge waveform is naturally lost, its repeated correlation with the step in the scattered light establishes conclusively that particle motion is responsible for the small charge or current pulses observed in all dielectric liquids under electric stress. The speed of the equipment used allows this correlation to be observed up to stresses of 46 MV m^{-1} in *n*-hexane.

The strong grounds for accepting that the waveforms relate to particle motion are not only that other possible mechanisms are not consistent with such well defined transitions, but that the shapes are invariant from low stresses, where particles can be visually observed, through the whole stress range.

These studies confirm, first, that particles are directly responsible for much of the randomly fluctuating component of current in stressed liquid dielectrics, but second, that the charge carried by individual particles is very low and corresponds to a potential of only $\sim 15 \text{ V}$ (refs 1, 2, 4). Perhaps even more significantly in terms of physical processes, they have not yet revealed any correlation between particle activity and the large (pC) pulses which have been observed by several authors to occur irregularly at high stresses. The ill-defined shape and duration of such pulses makes them difficult to study, even with the powerful on-line techniques mentioned above, and such a study is the object of our present work.

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Three modes of dissociation of H bonds in hydrogen-bond dominated solids

ICE, paper and regenerated cellulose, Nylon (unstretched and lightly stretched), and to a certain extent natural cellulose, lignin and wool are typical examples of hydrogen-bond dominated solids (whose mechanical properties are mostly controlled by the density and characteristics of the hydrogen bond). Previously¹ I have shown that for these materials, Young's modulus E is related to N , the effective number of H bonds per cm³ responding to unidirectional stress, by $E = kN^{1/3}$. For cellulose k is $\sim 8 \times 10^8$ when E is in Pa. Two major rheological characteristics of hydrogen-bond dominated solids are their ready softening by water and their relaxation of stress with time under constant strain. Thus, E_w/E_0 , the ratio of the modulus at w g H₂O/g solid to the modulus at $w = 0$, drops to very small values for paper at saturation. Similarly, E_t/E_0 , the modulus at time t to E at $t = 0$ in a stress relaxation experiment, drops to small ratios at high values of t . I postulate that both types of loss in E arise from reductions in N , through H-bond dissociation. I find this dissociation can be in one of three modes: I, II and III.

Mode I dissociation occurs on wetting a hydrogen-bonded solid within the range, $0 < w < w_c$ where w_c is a critical moisture content designating transition from mode I to mode II. w_c is postulated to be equal to w_m , the moisture content for a monomolecular layer calculated by BET equations¹⁷ applied to the adsorption isotherm of water on the particular polymer. Mode II occurs when $w_c < w < w_{sat}$ where w_{sat} denotes the water saturation value of w . Further, let N_0 be the value of N at $w = 0$ and W be the hypothetical value of w which allows one H₂O molecule for each potential H bond in the solid. (For cellulose, with potentially six H bonds existing per repeat unit of cellobiose, $W = 1/3$.)

In mode I we begin with

$$d(N_w/N_0)_1 = -(N_w/N_0)_1 d(w/W) \quad (1)$$

and arrive at

$$\ln(E_w/E_0)_1 = (1/3)(w/W) \quad (2)$$

(The suffix 1 denotes mode I.) Hence, specifically for cellulose

$$\ln(E_w/E_0)_1 = -w \quad (3)$$

This has been demonstrated for paper for $0 < w < w_m$ (ref. 1).

In mode II, dissociation is cooperatively undertaken by a number of bonds breaking down together. I use here Frank and Wen's hypothesis² of H bonds making and breaking cooperatively together in flickering clusters. Thus, I postulate that a molecule of water initiates the cooperative phenomenon by breaking only one bond, but this triggers others to break immediately. The total number is equal to the cooperative index (CI) but the reaction is still unimolecular in N . Thus

$$d(N_w/N_0)_2 = -(\overline{CI})(N_w/N_0)_2 d(w/W) \quad (4)$$

with initial condition at $w = w_c$

$$\ln(N_w/N_0)_2 = \ln(N_w/N_0)_1 = -(w_c/W) \quad (5)$$

Hence

$$\ln(E_w/E_0)_2 = (1/3) \{ (w_c/W) [(\overline{CI}) - 1] - (\overline{CI})(w/W) \} \quad (6)$$

For cellulose, $W = 1/3$, and equation (6) reduces to

$$\ln(E_w/E_0)_2 = w_c [(\overline{CI}) - 1] - (\overline{CI})w \quad (7)$$

The mean cooperative index ($\overline{CI}$) can be calculated from rheological data, but an *a priori* value was obtained from Starkweather's analysis³ of the cluster behaviour of water adsorbed on polymers, using Zimm's thermodynamic concepts^{4,5}, as well as calculations by Némethy and Scheraga⁶ for water at room temperature and found, for cellulose and Nylon 66, to be: $(\overline{CI}) = 6.71$.

Comparison of data in the literature on the dependence of E_w on w for paper⁷⁻¹¹ gives the mean for $(\overline{CI})$ as 6.70 ± 0.89 . The mean w_c for these experiments was 0.047 ± 0.011 , whereas w_m for paper is normally between 0.04 and 0.06. The average for different celluloses studied by Meredith¹² (ramie, Fortisan, viscose rayon and mercerised cotton) was found to be $(\overline{CI}) = 7.7 \pm 2.7$. The value for w_c was indeterminate because of the large standard deviations in the data, (-0.042 ± 0.09) . The average for Nylon 66 studied by Meredith¹³ and others^{18,14} was $(\overline{CI}) = 6.70 \pm 2.1$. The average w_c was indeterminate but clearly < 0.01 g H₂O/g solid (0.003 ± 0.005 ; W for Nylon 66 is 0.1593).

Thus, the cooperative dissociation of H bonds in mode II is still 'unimolecular' in N , even though approximately seven bonds are triggered to break down together on average.

In mode III, controlling stress relaxation under constant strain, the dissociation is polymolecular in N . This may be explained by imagining the need for a minimum number of bonds, α , having to dissociate simultaneously before a unit jump leading to stress relaxation becomes possible. Thus, it was found empirically from analysis of rheological data¹

$$\frac{d(N_t/N_0)}{dt} = - \sum_{i=1}^{i=\alpha} k_i (N_t/N_0)^{\alpha_i} \quad (8)$$

(where $N_0 = N$ at $t = 0$) with explicit interconnections between k_i with k_{i+1} and α_i with α_{i+1} respectively. The infinite series of different reaction rate constants k_i and cooperative indices α_i soon degenerate into a single reaction of rate k_1 and of order α_1 , the 'principal cooperative index', which controls the stress relaxation curve for all values of $(N_t/N_0) \lesssim 0.8$.

Analyses of data published by Houghton and Sellen¹⁵ on dry and wet regenerated cellulose at subzero temperatures reveal interesting maxima and minima in α_1 at particular temperatures.

Thus, in mode III the cooperative dissociation of H bonds is different from that in mode II in being truly polymolecular whereas in mode II it is unimolecular in N .

Details of these studies will be published elsewhere¹⁶.

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Cumulative deformation of magnesium oxide crystals by softer sliders

MECHANISMS postulated for the wear of hard crystalline surfaces by softer solids include thermal degradation¹, diffusion and solution²; and self abrasion of the harder solid when its wear debris is picked up by the softer surface³. A mechanism based purely on mechanical aspects has not yet been established but there has been the suggestion of an 'incubation period' during which the structure of the hard solid has been modified before the onset of visible wear⁴. In earlier publications^{5,6}, we have established that a dislocated zone was formed in some hard crystals by a softer slider, whenever the pressure between the contacting surfaces was greater than a certain threshold value which was thought to be related to the critical resolved shear stress. For a given load, and in these conditions, the depth of this zone was independent of the slider hardness—regardless of whether the point itself was blunted or visible macroscopic deformation and penetration of the harder crystal was obtained. Then, the depth of the dislocated zone was determined only by the magnitude of the applied load. There were two principal objectives to this particular study. First, to measure the effect of slider hardness on the number of traversals necessary to initiate visible fragmentation and wear in the harder crystal. Second, to use dislocation etching and microhardness techniques to follow the deformation of the harder crystal during the microdeformation preceding fracture—the incubation period.

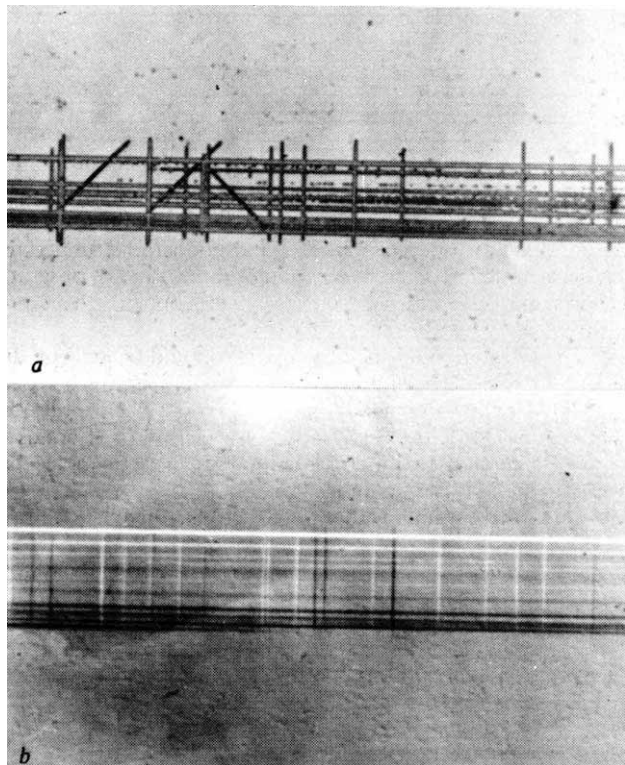


Fig. 2 Stages in the deformation produced by a lubricated slider, under a normal load of 500 g, making traversals in a [100] direction on the (001) plane of MgO. *a*, Etch pits revealing dislocations after first traversal, ($\times 160$). *b*, Slip lines visible after ten traversals. ($\times 160$). *c*, Fragmentation and wear marking the end of the experiment at $N_c = 15,000$. ($\times 24$).

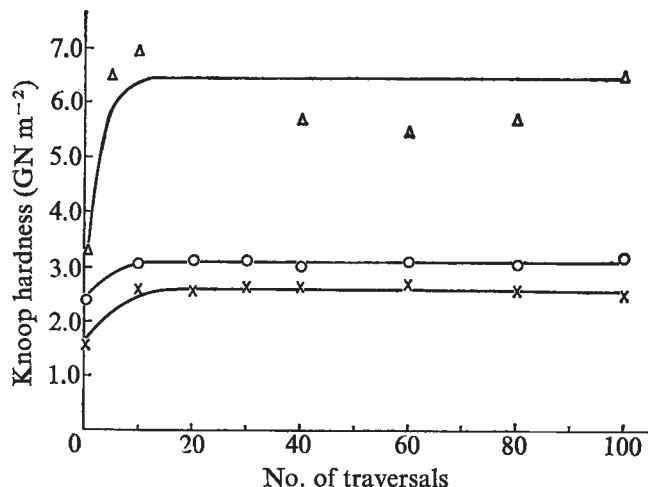


Fig. 1 Illustrating the degree of work hardening, as the tip of the slider was blunted, during the initial traversals on MgO, for Δ , EN 58J; $\circ$, 95/5 phosphor bronze; $\times$, 70/30 brass.

The apparatus used has been described in detail elsewhere and only brief experimental details will be given here. All the following results are based on experiments using magnesium oxide as the hard crystal with conical sliders, having included angles of 136° at the start of the experiment, made from polycrystalline alloys. The alloys selected for this purpose included an austenitic stainless steel (EN 58J), an α brass (70/30), and a phosphor bronze (95/5). Experiments were carried out at room temperature, using reciprocating sliding at a speed of $3 \times 10^{-4} \text{ m s}^{-1}$, and the surfaces were continuously lubricated with soluble or mineral oils. The direction of sliding was aligned with $\langle 100 \rangle$ crystallographic directions on the chemically polished (001) plane of MgO and the length of the resultant wear track was $\sim 0.01 \text{ m}$. Normal loads of 0.5, 1 or 2 kg were used.

The first few traversals caused a flattening of the tip on the metallic slider with a corresponding increase in hardness from conventional work-hardening processes. This effect is reflected in microhardness measurements on the blunted tip, using a Knoop indenter with a normal load of 100 g, as a function of the number of traversals (Fig. 1). The hardness of the slider tended to a constant value, presumably indicating the ultimate level of work hardening for that particular material, within the first ten cycles. There was no visible damage on the MgO surface after the first traversal but dislocations in the contact zone, induced by the sliding, were revealed by etching (Fig. 2*a*). Subsequent traversals produced slip lines on the MgO surface—typically within the first ten cycles (Fig. 2*b*). Eventually, a visible crack was formed and then was immediately followed by significant fragmentation (Fig. 2*c*). The crack pattern was of the chevron form, characteristic of rock salt crystals, produced by fracture on $\{110\}$ and $\{100\}$ planes⁷. The number of traversals to cause the initiation of the first crack, and thus fragmentation, was related to the ultimate hardness of the slider material. The harder the slider, the fewer the number of traversals (N_c) required to produce a crack.

A plot of the ultimate hardness of the slider (H_s) against the critical number of traversals to fracture (N_c) gives a curve which resembles that of an $S:N$ curve based on conventional fatigue data (Fig. 3) in which the maximum stress applied by the slider in each traversal is directly related to the hardness of the slider material. It is important to note, in this context, that the critical number of cycles was independent of the normal load for a given slider. Also, that directly comparable results were obtained with a mineral oil and a water-soluble lubricant. Thus it would appear that this particular change in environment is not important in the wear mechanism. Further support for a mechanism based on fatigue criteria was given by an experiment in which an MgO surface was abraded with a brass for $\sim 12,000$ cycles—that is, well after maximum work hardening of slider and crystal but before the onset of fragmentation and wear. Subsequently, the top 5 μm of the dislocated zone, which penetrated the crystal to a total depth of 150 μm , was removed by chemical polishing. Sliding was then continued, along the same track, and a further 15,000 traversals were required to produce fragmentation and wear. This compared with $N_c = 15,000$ for the original, undeformed, surface. Note that each point on the curve in Fig. 3 represents the average of at least six measurements, all within a range of 10%.

The coefficient of friction (μ) was measured throughout the experiments for all three types of slider. Where the lubricant became ineffective, adhesion caused a marked increase in friction and fragmentation was immediately observed. Results from such experiments have not been included in Fig. 3. In the normal case, μ decreased during the initial mutual work hardening of slider and crystal but then remained constant until the onset of fragmentation. At this point, an immediate and significant increase in friction was observed. It can, however, be concluded that fragmentation was responsible for the increased friction and not vice versa.

Examination of the abraded MgO surface, using dislocation etch pit techniques and microhardness measurements, confirmed some of the earlier observations made on the basis of a single

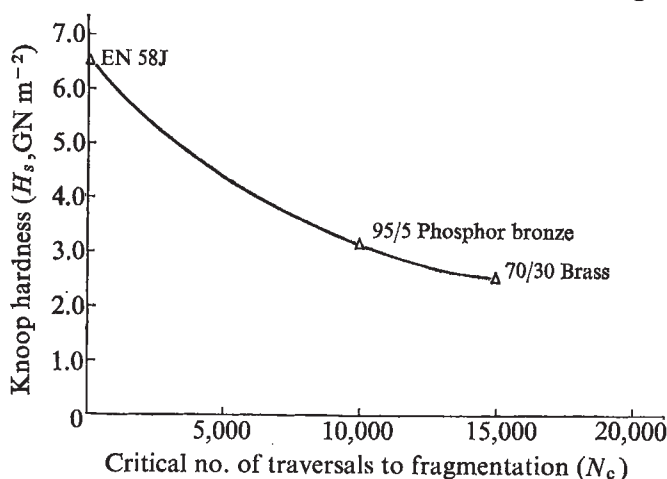


Fig. 3 The $H_s:N_c$ curve for a (001) MgO surface abraded in the [100] direction. Note a limiting slider hardness of $\sim 2.25 \text{ GN m}^{-2}$.

indentation or traversal⁵ and gave further information on the nature of the incubation period. The depth of the resultant dislocated volume was determined not by the shape or hardness of the slider but only by the magnitude of the applied load. Dislocations produced by repeated traversals were generally contained within the dislocated volume established by the first traversal. Thus the dislocated volume remained constant whilst the dislocation density increased during the first hundred traversals. Furthermore, microhardness measurements show that the increase in dislocation density causes work hardening both on the surface and within the bulk of the magnesium oxide. Typical values, using a Knoop indenter aligned in a [110] direction on the (001) MgO surface with a load of 100 g, are shown in Fig. 4 for all three slider materials. It would seem that

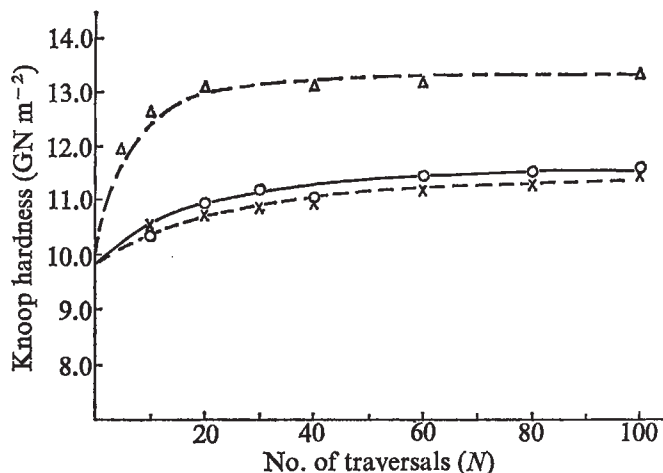


Fig. 4 Hardening of the MgO surface, by traversals on the (001) plane in a [100] direction, was shown to be dependent on the ultimate hardness of the slider material. Δ , EN 58J; $\circ$, 95/5 phosphor bronze; $\times$, 70/30 brass.

the degree of work hardening in the MgO is again related to the ultimate hardness of the slider material. Results obtained by removing the surface layers, using chemical polishing techniques, and indenting on the newly exposed surfaces in the same manner reveal the hardening in depth below the original surface (Fig. 5).

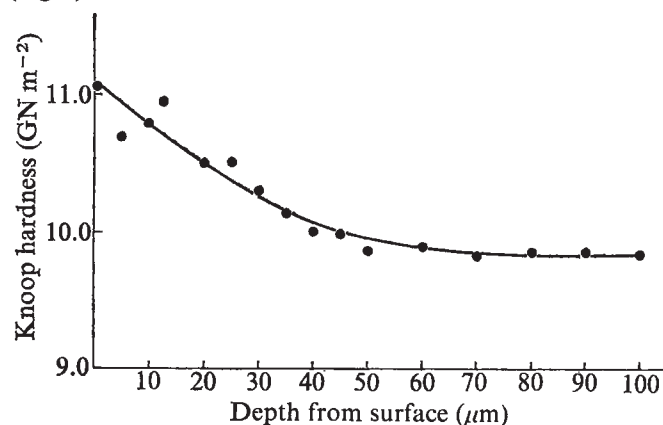


Fig. 5 Bulk hardening of the MgO crystal after ~ 200 traversals of brass slider under a load of 500 g.

In summary, these experiments provide a method of identifying the range of slider hardness within which fragmentation and wear of a harder crystal can be anticipated. Also, we now have the possibility of establishing a limiting slider hardness, akin to a fatigue or endurance limit, below which fragmentation of a given solid will not take place in a given lifetime. For example, MgO with a hardness of 9.8 GN m^{-2} should have a reasonable lifetime, in abrasive conditions, provided the hardness of the softer material does not exceed 2.25 GN m^{-2} . Current work has established that the behaviour of various other non-metallic crystals is comparable with MgO and similar $H_s:N_c$ curves have been obtained. Clearly this information is of technological interest wherever a soft material abrades a harder one—as in gemmological applications and metal cutting operations.

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Feathering and flight evolution in *Archaeopteryx*

WITH the description of further specimens^{1,2} there has been a resurgence of interest in *Archaeopteryx*. Although there is no final agreement on whether it was mainly arboreal or terrestrial, recent opinion³ seems to favour the latter. There has been discussion on its reptile origins⁴⁻⁶ and on its ability to fly and the general problem of the evolution of powered flight^{3,7-12}. However, most of these studies have been made by specialists who are not ornithologists and who usually tend to extrapolate from reptile forms and behaviour in their discussions. Whatever its precursors, *Archaeopteryx* is regarded as a bird and to interpret adequately such evidence as we have we must consider it in relation to what we know of birds as well as reptiles.

Archaeopteryx possessed structures very closely resembling the flight feathers of Recent birds, which should confer an advantage by supporting it during movement, while body feathers, if present, would aid thermoregulation; but feathers also have disadvantages in being fragile and subject to abrasion. In Recent birds this is partly overcome by regular moult and replacement, but we do not know if *Archaeopteryx* had a moult. Recent birds also avoid feather damage by postures and movements in which the plumage does not usually rub or strike against surrounding objects, the larger feathers of wings and tail usually being held clear of the substrate. The possession of feathers by *Archaeopteryx*, even were it a reptile, should therefore impose some limitations on both posture and movement, comparable with those of Recent birds; and these limitations would be likely to have modified some of its characters already, and to affect its subsequent evolution.

Feathers probably evolved before avian flight and Ostrom³ has suggested that the large feathers of the forelimbs were originally produced for a different purpose. He regards the digits as functional in catching small live prey and suggests that the feather may have formed a large area of catchment for trapping small creatures such as insects. It is possible to envisage an earlier and shorter form of feathers being spread like small nets and slapped down over insects to aid capture, but I would suggest that the feathers on *Archaeopteryx* had developed beyond this stage and that to use them for such a purpose would be likely to damage and destroy them, while the total size of the spread wing would tend to hinder the use of the forelimbs for rapid movements in catching prey.

It cannot be argued that the similar large feathers along either side of the tail were used for capturing prey, and in view of their position it is reasonable to suppose that their function was for support during movement. It is also likely that the extensive feathering of the forelimbs had a support function at this stage in evolution, and while Ostrom^{3,12} argues that the skeletal structure precludes an ability to fly in the true sense, there seems to be no objection to the idea that the feathers of forelimbs and tail might be used to prolong the passage through the air of an individual that had leaped from some eminence, whether by gliding or by some crude flapping flight.

The feathering should affect both normal movement and the subsequent evolution of flight. Some earlier reconstructions of *Archaeopteryx* and theoretical proavian forms based on Recent reptiles^{13,14} assumed that *Archaeopteryx* climbed trees by using the well developed digits, and launched itself into the air from a height; with flight evolving through a stage of gliding down from high perches. The feathering of *Archaeopteryx* is developed to a degree

that makes it most improbable that it would climb in a lizard-like fashion or clamber among twigs using all four limbs as some reconstructions suggest. Any arboreal movement is likely to have been limited to perching, running along branches and leaping between them, meanwhile probably using the wings for balance and aerial support.

In his recent papers Ostrom^{3,6,12} describes *Archaeopteryx* as a cursorial, and presumably terrestrial predator. This would accord with the above comments but would not explain why powered flight should have evolved in such circumstances. He believes the flight of *Archaeopteryx* to have been "flapping leaps" to capture insect prey, and presumably envisages flight evolving as an aid to predation. In Recent birds, flight as a means of catching prey appears to be a secondary adaptation, for species which use it, from simple flycatchers to the more elaborate aerial feeders, have additional structural modifications for this purpose, and in its earlier stages flight would have had the greatest value as a means of escape from predators. Driver and Humphries¹⁵ in their studies of protean behaviour have shown that an element of unpredictability in the behaviour of a prey species will help it to evade capture. If prey which has previously moved along the ground suddenly launches itself into the air in a long leap, leap and glide, or full flight, a terrestrial predator is temporarily baffled and likely to discontinue the hunt. This is a very effective anti-predator device, a fact reflected in the evolution of such behaviour in a number of different groups of animals. Anyone who has tried to catch an almost-fledged nestling or a slightly injured adult bird without the powers of sustained flight will be aware of the way in which even brief bursts of short flight by the prey can make capture difficult for a relatively sophisticated predator such as man.

We can envisage *Archaeopteryx* as a bird-like creature probably resembling the coucals, *Centropus* spp., which are similar in general proportions with rather weak rounded wings, long tails and well developed legs. These latter birds are mainly terrestrial or semi-arboreal, walking or running through low vegetation or travelling through shrubs and low trees, moving along branches and jumping from branch to branch. They fly with reluctance but if hard-pressed will ascend to a higher perch and then take off in a weak and frantic flight, travelling a little distance before dropping into low vegetation and disappearing.

Archaeopteryx could have moved in similar fashion, and if launching itself from some eminence enabled it to escape terrestrial predators, there would have been a selective advantage in a readiness to take flight. If the ability to move through the air was poor and likely to result in a landing too close to the point of take-off there could be a likelihood of rediscovery during random searching by the predator. There would therefore be additional selection pressure in favour of individuals that could travel further through the air before landing. It can be argued that factors such as these could provide pressures for the evolution of powered flight in a mainly terrestrial animal without the need for an ability to climb in the strict sense or for the modification of specialised predatory leaps.

Regarding *Archaeopteryx* as a bird is therefore useful in predicting the likely limitations implied by the possession of feathers, and the probable advantages of even brief periods of movement in the air. It may prove helpful in interpreting other aspects of this important evolutionary link.

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Intake and digestion of hill-land vegetation by the red deer and the sheep

DOMESTICATED sheep and feral red deer (*Cervus elaphus*) are the two principal producers of food for human consumption from a large proportion of the rough grazings of Scotland. There is considerable interest in the possibility of increasing the production of venison by farming the red deer, and studies of the degree of domestication required, the development of biologically sound management practices and their economic viability are in progress¹. A fundamental question is whether the red deer can utilise the indigenous vegetation found on the hills more efficiently than does the sheep, and, if so, to what extent this is brought about by its greater ability to range over and graze selectively on hill vegetation or by its ability to consume and digest more of the poor quality vegetation. We have investigated this latter aspect and now report that on such diets red deer consume greater quantities of digestible dry matter per unit metabolic body weight than sheep. The greater ability of the deer to increase voluntary food intake during the summer months is also discussed.

For this study we used 12 castrated male red deer and 12 castrated male Scottish Blackface sheep, aged 18 months. Both species were treated in a similar manner from 6 months of age and were accustomed to being housed in metabolism pens before being given diets of common heather (*Calluna vulgaris*, L. Hull) and *Agrostis/Festuca* grass heath (two of the common types of hill vegetation grazed by both species). In two experiments carried out in January and April 1976, the animals were offered, *ad libitum*, freeze-stored vegetation which had been harvested mechanically in the autumn and which was known to provide a diet similar to that selected by grazing during the winter (unpublished data of J.A.M.). Measurements were made of the voluntary food intake (VFI) and digestibility of each diet. The particulate-phase marker, ¹⁰³Ru-phenanthroline², was used to estimate

the mean retention time³ (MRT) of digesta between the rumen and the faeces—an index of the time undigested food particles spend in the gastrointestinal tract. Results are given in Table 1.

In both January and April the red deer ate at least twice as much heather and *Agrostis/Festuca* as the sheep. Surprisingly, on the heather diet the deer were able to digest their higher intake to the same extent as the sheep (Table 1), but with the *Agrostis/Festuca* diet they showed a more predictable fall in digestibility (7–8 percentage units). On both diets MRT was considerably shorter for the deer than the sheep. It is not possible to assess to what extent these differences were due to the different intakes of the two species, but further experiments have shown that when the same animals consumed similar quantities of a higher quality ration (dried grass pellets), the MRT in the red deer was still significantly ($P < 0.001$) shorter than in the sheep (unpublished). This latter result is in agreement with earlier studies using chopped dried grass and grass hay diets^{4,5} which suggest a real between-species difference in the speed at which digesta passes through the digestive tract.

The VFIs of both the red deer and the sheep were higher in April than in January. In the sheep, the 40% increase in VFI of heather in April was accompanied by a reduction in the digestibility of the diet and in MRT. This result is in agreement with relationships between VFI, digestibility and MRT previously found in sheep and cattle and which support the generally accepted hypothesis that with medium-quality long roughage diets, the control of VFI in sheep is related mainly to the filling effect in the digestive tract, particularly in the rumen, and to the rate of removal of food residues from that organ⁶.

In contrast, the large increase in VFI of deer of 70% between January and April, which has been associated with changes in daylength⁷, was not accompanied by any change in digestibility or MRT. This suggests that the amount of feed residues transferred per unit time down the gastrointestinal tract of the red deer was higher in April than in January. The hypothesis that VFI of long roughages is controlled by the filling effect in the digestive tract can only hold for the deer at both times of the year if the volume or capacity of their digestive tract, or at least some section of it, had increased considerably. Although the mechanisms whereby this may occur are not known, it is likely that they are under some form of hormonal control related to day-length.

In spite of these seasonal differences in VFI the red deer ate twice as much digestible dry matter as the sheep on both kinds of hill vegetation. This suggests that red deer are better adapted than sheep to grazing hill vegetation in winter and spring, providing that both species exhibit similar grazing behaviour, have similar maintenance requirements per unit of metabolic liveweight, and have similar rates of production and utilisation of the end products of digestion. These latter aspects are being investigated further.

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Table 1 Intake and digestibility of diets

		DMI	DMI/ kgW ^{0.75}	DMD	MRT
		(g)	(g)	(%)	(h)
January					
Red deer	<i>Agrostis/Festuca</i>	1,199	49.1	40.1	27.4
	Heather	847	35.0	45.3	37.7
Sheep	<i>Agrostis/Festuca</i>	432	24.1	48.8	56.1
	Heather*	327	17.7	45.7	67.3
April					
Red deer	<i>Agrostis/Festuca</i>	2,028	76.2	41.9	32.5
	Heather†	1,446	56.6	45.2	42.4
Sheep	<i>Agrostis/Festuca</i> †	485	26.6	48.1	55.9
	Heather	458	24.5	42.8	56.2
s.e.m.		±74.9	±2.87	±1.06	±3.33

*Mean of four observations.

†Mean of five observations.

Figures show mean daily voluntary intake of dry matter (DMI), dry matter intake on metabolic liveweight basis (DMI per kgW^{0.75}), dry matter digestibility (DMD%) and mean retention time of a particulate-phase marker (MRT) for red deer and sheep offered diets of *Agrostis/Festuca* and common heather in January and April (means of six observations)

⁴ Maloij, G. M. O., and Kay, R. N. B., *Q. Jl exp. Physiol.*, **56**, 257–266 (1971).

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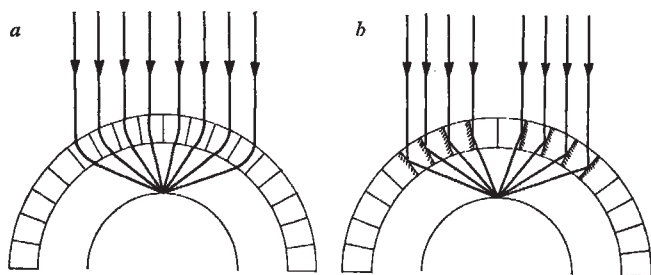
Superposition images are formed by reflection in the eyes of some oceanic decapod crustacea

LIKE moths, the eyes of many pelagic and bottom-living decapods appear to glow brightly when viewed from the direction of illumination¹. It has generally been assumed that the optical system involved is similar to that in nocturnal insects, that is, the superposition mechanism of Exner². The essential feature of Exner's theory is that the optical apparatus of the cornea and crystalline cone refracts light through twice the angle at which it was incident on the cornea (Fig. 1a). Light from many facets then combines to form a single image on the receptor layer, and if this is backed by a tapetum the light is reflected out through the same facets that it entered, causing the glow. There is now no doubt that Exner's mechanism is present in some insects³. Recent accounts have, however, cast doubt on whether it occurs in crustacea^{4,5}. This is largely because the high refractive indices and inhomogeneities required of the optical components have been shown not to be present. In this paper I show that superposition images are formed in the eyes of a midwater shrimp, but in a different manner (Fig. 1b).

Live *Oplophorus spinosus* (Coutiere), about 5 cm long, were obtained from trawls at a depth of 500 m in the North Atlantic during a recent cruise of the RRS Discovery (July–August 1976). The eyes are almost spherical, and when illuminated they exhibit a bright orange glow over a circular area whose diameter (905 μ m) is slightly more than half the diameter of the eye itself (1,580 μ m). The glow does not disappear on exposure to light, indicating the absence of occlusory distal pigment such as is found in moths⁶. After fixation for 1 h in a mixture of glutaraldehyde and formalin in cacodylate-buffered seawater, which stiffens the tissues without loss of transparency, an eye was cut in half, and the layout of the component parts is shown in Fig. 2b. As in insect superposition eyes, the rhabdom layer has a radius of curvature approximately equal to half that of the eye surface.

A superposition image was clearly visible when the half eye was illuminated by a small source (a light guide) at a distance of 10 cm. This produced a single small but somewhat diffuse spot of light which moved around the rhabdom layer in the same direction as the source—that is, the image was erect. Occluding different parts of the cornea showed that light contributing to the image entered the eye over approximately half its diameter. When two sources were used distinct images were observed, provided the sources were separated by 15° or more at the eye.

Fig. 1 Superposition image formation (a) by refraction (modified from Exner) and (b) by reflection.



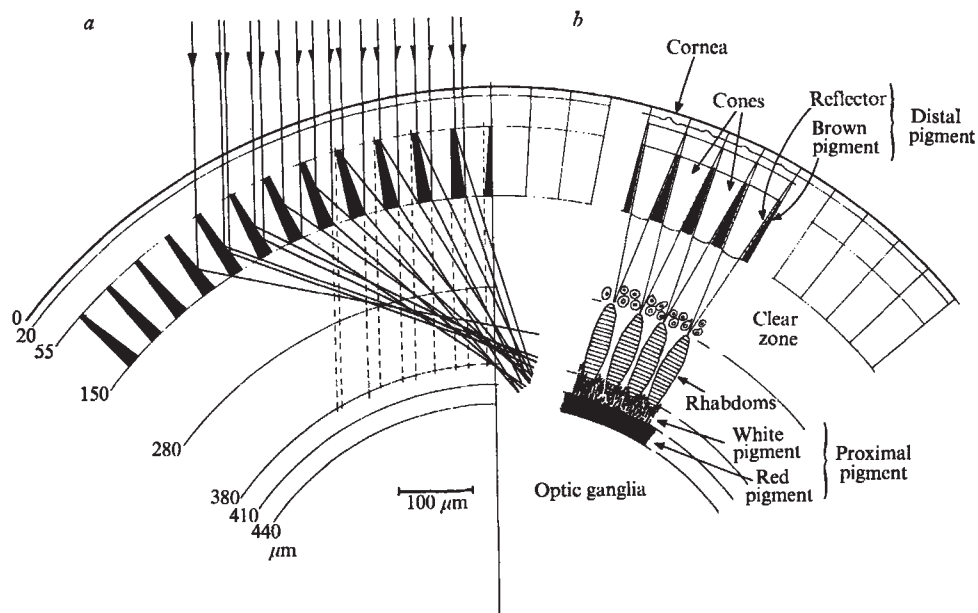
Dissection of unfixed material failed to reveal any 'crystalline' cones, although clear gelatinous material was present in the spaces in the distal pigment region, and also in the 'clear zone' between this and the rhabdoms. This is in marked contrast to the situation in, for example, euphausiid eyes where hard highly refractile cones are present as a layer beneath the cornea. The corneal facets themselves have negligible focusing properties. After fixation the gelatinous material stiffened into recognisable square-sided tapering structures (Fig. 2b) which could be easily separated from each other. Each of these consisted of a short non-pigmented outer section, a second more refractile segment extending to a depth of 150 μ m from the eye surface, and lined with pigment for the proximal two-thirds of its length, and a long tapering tail whose tip lay 280–300 μ m deep, in the layer of cell bodies immediately above the rhabdoms. An estimate of the refractive indices of the parts of fixed cones was made by finding cones lying with their side faces at 45° to the microscope slide, and measuring the prismatic bending of parallel rays passing through them. The distal region gave a deviation of 5.9°, corresponding to a refractive index of 1.41, and the proximal tail gave 3.3° or an index of 1.37. These values are quite in keeping with the consistency of the material, and are far too low to be compatible with image formation by refraction, using curved surfaces or lens-cylinder optics. (Euphausiid cones, treated similarly, gave a refractive index of 1.54.)

Teasing fresh material from the distal region showed that the cones are separated from each other by two kinds of pigment: a layer of reflecting material which lines the four faces of each cone and has a green specular appearance at near normal incidence, and behind this a brown pigment which fills the gap between the cones. Optically, therefore, each cone can be thought of as a silvered, four-sided prism.

This observation leads directly to the hypothesis of image formation outlined in Figs 1b and 2a. Parallel light reaching the eye is reflected at one of the faces of each cone and is bent through an angle approximately equal to twice the angle of incidence at the eye, just as in a refracting superposition eye (Fig. 1). The angle is in fact slightly greater than this, because of the 8° taper of the cones. A consequence of this is that many facets will redirect light to an approximate focus on the surface of a sphere concentric with the eye, and with slightly more than half its radius of curvature. The limiting ray will be the one that just manages to enter and emerge from a reflecting prism (left-hand ray, Fig. 2), and this makes an angle of 60° with the eye surface. This will also be the ray which emerges nearest the edge of the patch of 'glow' and it will be located approximately half way out from the centre of the eye. It can be seen from Fig. 2a that some light will tend to pass straight through the aperture of the central cones without being reflected (dashed lines). The fate of this light is uncertain: some will certainly be totally internally reflected by the walls of the tails of the cones (angles of incidence more than the critical angle, 77°) and the image is not likely to be greatly spoiled.

Two other features of the glow tend to confirm the hypothesis just given. Towards the edges of the glow patch light from individual facets is unevenly distributed, each showing a dark band which gets wider and finally occludes the facet completely. This 'venetian blind' effect is simply explained by the progressive restriction in the width of the slit of light that can enter and emerge from each reflecting prism (Fig. 2a). Secondly, the colour of the glow changes from yellow/orange in the centre (that of the bleached rhabdoms) to green at the edge of the patch. If, as seems likely, the mirror surface is due to thin-film interference⁷, one would expect that all wavelengths would be reflected at high angles of incidence (near the centre), but that the light would take on progressively the interference colour of the

Fig. 2 Optical (*a*) and anatomical structure (*b*) of part of the eye of the shrimp *Oplophorus*. In *a* it is assumed that the only optical components in the eye are the reflector-lined faces of the cones. Solid lines show how reflected light is brought to a focus, and dashed lines indicate pencils of rays that pass through directly. The figure was constructed as accurately as possible from measurements made on fresh or recently fixed eyes. The numerals on the left show depths of each layer from the surface. *b* shows the parts of the eye as visible in a hemisected eye seen through a dissecting microscope.



reflecting material itself at lower incident angles. Where this angle reaches 60° , at the edge, the green colour observed is similar to that of the reflecting material observed directly.

It is concluded that reflection in the cones provides an adequate explanation of the images observed, and that a refraction mechanism is not present. Since this paper was first submitted, I have learned from Professor K. Kirschfeld (Tübingen) that exactly the same optical arrangement as that given here has already been proposed independently by Klaus Vogt (Stuttgart) to account for image formation in the eyes of freshwater crayfish⁸, which also exhibit a 'glow' when dark adapted⁹. His note was published last year. It also turns out that the theoretical possibility of imagery of this kind was in fact dismissed by Exner in this 1891 monograph, and the discovery of this mechanism as a practical reality properly belongs to Vogt. His paper and mine leave little doubt that this is the standard method of superposition image formation in decapod crustacea.

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For example, if the rarest genotype is most fit then the rare allele will increase in frequency but will not become fixed in the population, because as it gets more common the fitness of its carriers decreases. This mechanism is attractive since it leads to a stable gene frequency equilibrium which may not involve problems of genetic load or inbreeding depression. Experimental evidence has been obtained^{8,7} which supports the view that frequency dependence is involved in the maintenance of the esterase-6 (*Est-6*) and alcohol dehydrogenase (*Adh*) loci in *Drosophila melanogaster*. However, these results are subject to two major criticisms⁸⁻¹¹. First, the observed frequency dependence may have resulted from poor experimental design. In the original report⁸ fitness estimates were made by comparisons of adult frequencies with those expected from the gene frequencies in the previous generation. Frequency-dependent selection appears as an artefact of this method¹². Second, since inbred lines were used in these studies problems of linkage disequilibrium may have occurred. If this view is correct then the observed selective differences were due to differences between lines, not to differences between alleles at a locus. As a consequence of these criticisms the role of frequency dependence as a force responsible for maintaining enzyme polymorphism has been disputed⁹. The experiments reported here overcome these criticisms and indicate that frequency-dependent selection should be reconsidered as an important mechanism involved in the maintenance of enzyme polymorphism.

From the Kaduna population, two pairs of lines were derived. One pair were segregating at the *Adh* locus while homozygous at the *Est-6* locus for the *F* and *S* alleles respectively whereas the other pair were homozygous at *Adh* and segregating at *Est-6*. Each line was derived independently as the progeny of 30 pairs of homozygous parents sampled from the population. The lines had been kept in population cages (as large populations) for approximately 18 months before the experiments were carried out. For each experiment 200 newly-emerged larvae (0–3 h old) were competed. They were put in a 10×4-cm *Drosophila* vial containing 10 ml of the following medium: maize meal, 26.25 g; molasses, 26.25 g; agar, 10 g; flaked brewers yeast, 2.5 g; nipagin, 2.5 g; propionic acid, 2.5 ml; water, 1,000 ml. The frequency of the *F* allele in the experimental vials was altered by changing its relative proportion in the larval mixture (for example, a vial with $P_F=0.15$ would contain 30 *FF* homozygotes and 170 *SS* homozygotes). For the *Est-6* experiment seven frequencies of the *F* allele were used ($P_F=0.15; 0.25; 0.40; 0.50; 0.60; 0.75; 0.85$) and for the *Adh*

Frequency-dependent selection at two enzyme loci in *Drosophila melanogaster*

RECENT development in population genetics indicate that it is often misleading to treat selective values as constants¹⁻³. These studies indicate that selective forces may be a function of genotype frequencies (frequency-dependent selection).

Table 1 The relative viabilities of *FF* and *SS* flies when grown at different frequencies

Enzyme locus	P_I	<i>FF</i> input*	<i>SS</i> input*	<i>FF</i> survived (%)	<i>SS</i> survived (%)	Total survived (%)
	0.15	120	680	55.8	34.0	37.3
	0.25	200	600	46.5	38.0	40.1
	0.40	320	480	40.9	46.9	44.5
<i>Est-6</i>	0.50	400	400	38.8	43.0	40.9
	0.60	480	320	27.1	46.9	35.0
	0.75	600	200	38.0	57.0	42.8
	0.85	680	120	33.2	68.3	38.5
	0.15	120	680	65.8	45.7	48.8
<i>Adh</i>	0.50	400	400	43.8	35.3	39.5
	0.85	680	120	53.4	52.5	53.3

*Each result is the sum of four pooled replicates.

experiments three frequencies of the *F* allele were used ($P_I=0.15$; 0.50; 0.85).

The genotypes of all emerging flies were determined by the methods of O'Brien and MacIntyre (*Est-6*)¹³ or Day *et al.* (*Adh*)¹⁴. The results are shown in Table 1.

The results have been analysed by the heterogeneity χ^2 method (Table 2). The regression of the difference in survival between *FF* and *SS* flies on frequency is significant for both the *Est-6* and *Adh* experiments (*Est-6*, $P<0.005$; *Adh*, $P<0.01$). These results clearly demonstrate frequency dependence operating at both the *Est-6* and *Adh* loci. For the *Est-6* locus at *F* frequencies less than about 30% more *FF* flies survive than *SS* flies. This situation is reversed at frequencies above about 40% *F*. Here it would seem that a frequency-dependent mechanism is sufficient to maintain the polymorphism, at least in these culture conditions. This is not the case for the *Adh* polymorphism since more *FF* flies survive than *SS* flies at each frequency. The results for the *Adh* polymorphism differ from those of Kojima and Tobari⁷ since these authors presented data which showed that the relative advantage of the *F* allele changed to a disadvantage as its frequency increased. My results have been confirmed by Briscoe¹⁵ who used the same materials but different experimental methods. In a further communication (Morgan, in preparation) I will present data that indicates that the degree of frequency dependence at the *Adh* locus can be modified by growing flies on different culture media.

The results presented in this paper suggest that the role of frequency dependence in maintaining enzyme polymorphism should be examined in greater detail.

Table 2 Heterogeneity χ^2 of the differences in survival between *FF* and *SS* flies grown at different frequencies*

	d.f.	χ^2
<i>Est-6</i>		
Average difference in survival between <i>FF</i> and <i>SS</i>	1	29.66*
Linear regression of S_I-S_S on P_I	1	99.00*
Departure from linearity	5	9.25†
Replicates	21	23.74†
<i>Adh</i>		
Average difference	1	14.86*
Linear regression	1	7.54†
Departure from linearity	1	0.17†
Replicates	9	4.15†

For each trial, differences in survival between the two genotypes may be tested as χ^2 , which equals $nP_I(1-P_I)(rS-S_S)^2/S(1-S)$, where n is the total number of individuals at risk, P_I is the starting frequency of *FF* flies and S_I , S_S and S respectively are the proportions of *FF*, *SS* and all flies which survive. Over a set of trials, the sum of these values may be partitioned as in the table.

* $P < 0.005$.

†No significant difference: $P > 0.05$.

‡ $P < 0.01$.

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X-ray induced mutations, DNA and target theory

THAT X-ray induced, specific locus, germ-line mutation rates vary significantly in eukaryotes is generally known, but the factors that are responsible for such differences have not been assessed in detail. It was recently proposed¹ for a variety of species that mutability is closely and simply related to total DNA per genome. This approach, however, has not stood the test of critical review²; in fact, even within one species (mouse) there are major differences between the rates for different genes, and even for different germ cells³⁻⁵. I have therefore taken a different approach by considering the radiobiology of the individual gene. The analysis leads to three major conclusions. (1) Mutation rates tend to be much lower than radiation theory predicts. (2) Selection and/or repair are major factors that determine the rates. (3) The mouse 7-locus test, which provides a principal data base for the standards of human radiation hygiene, may not provide adequate overall representation of the mutability of the mammalian genome, so more research is needed.

Before considering the differences in rate between organisms, it is of interest to examine the differences between genes that are irradiated in the same germ cell of a particular organism. As an elementary step, consider the following analysis that is based on classical target theory. The fundamental premise is that bigger genes (molecules) are more likely to be hit than smaller ones and therefore should be more mutable. The calculation of target size involves the relation between observed hits and the distribution of ion pairs generated in the exposed material by the absorbed radiation, and has been applied to the study of enzymes and viruses^{6,7}.

In the usual germ-line mutation experiment, a parent is irradiated and the F_1 progeny are screened for the mutant phenotype. The mutation rate of a particular gene is calculated as mutations per rad per generation where per generation indicates that the observations were made on the viable progeny. But the frequency of observed hits (mutants) in these progeny is unlikely to be equal to the frequency of hits (mutations) in the parental germ cell since: (1) some hits will be repaired; (2) some hits will be carried in cells that die out or are selected against; (3) some hits will induce trivial mutations, that is, the mutation does not induce a significant change in phenotype although

provided adequate opportunity to do so. These "deflating" factors will tend to make the target-theory estimate of gene size too small.

There are also "inflating" factors that tend to make the estimate too large. First, in addition to direct hits, free radicals generated in nearby molecules (for example water) may mutate the target gene. Secondly, since "target" is an operational term, its volume might in fact represent the aggregate volume of several separate genes, any one of which when hit can directly or indirectly induce the mutant phenotype.

It is clear that the estimate of gene size by target theory will probably be incorrect, depending on the magnitude and balance of a number of factors. Nonetheless, such an incorrect estimate can be useful for purposes of comparison. As the standard for such comparison, we use the molecular weight of a typical gene. Since each codon has a molecular weight of about 2,000, a gene that determines a protein of 200 amino acids has a molecular weight of about 400,000.

I applied target theory to the data of the three tests with mouse spermatogonia (250-kV X rays, 60–90 rad min⁻¹), using the method outlined by Lea (chapter 3, fig. 9 of ref. 6). The method is adequate for the comparisons to be made here, although it does not provide a full treatment of the problem^{8–10}.

The 7-locus test used one cartilage and six coat-colour genes. For a dose of 300 rad, the mean probability K of inducing a mutation was 2.7×10^{-7} per gene per rad per generation⁴. Greater doses or lower dose rates reduced the probability. The mean dose per mutation (Lea's 37% dose) is therefore taken as $1/K = 3.7 \times 10^6$ rad, for which the associated molecular weight is 360,000 (180 codons). The close agreement of this estimate with that based on the model is presumably fortuitous. Nonetheless, the fact that there is numerical agreement rather than gross disagreement gives perspective on the balance of deflating and inflating factors.

In a very much smaller set of experiments with an analogous "6-locus test", the value of K was about one-third that of the 7-locus test¹¹.

In the case of the H-test (30 class I histocompatibility genes)⁵, the mean value of K was 1/60 that of the 7-locus test, and the associated molecular weight is therefore about 5,000—an underestimate equivalent to 2.5 codons. The underestimate is informative, however. It indicates that the set of deflating factors is much more important than the inflating ones.

With two such disparate results for the mouse as a point of departure, their generality becomes of interest—will various genes in diverse species behave like the 7-locus group or the H-group? The literature was recently reviewed² for X-ray induced, intralocus, forward mutation rates. Determinations were found for *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (yeast), *Neurospora crassa* (mould), *Drosophila melanogaster* (fruit fly), *Arabidopsis thaliana* (flowering plant), *Zea mays* (maize) and *Hordeum vulgare* (barley). From the summary, I obtained the distribution of 16 values of K for different genes and different groups of genes. The highest value was 4.7×10^{-8} , equivalent to 25 codons. Eleven fell between 3×10^{-8} and 4×10^{-9} , with a mean of 1.5×10^{-8} (7 codons), and four were below 3×10^{-9} (1 codon).

It seems that the genes or groups of genes tested so far (outside the mouse) tend to react like the mouse H-group rather than the 7-locus group, and that the factors that deflate the mutation rate (per gene per generation) are much more effective than those that inflate it. To what extent this conclusion will continue to be generally true for all types of genes, and especially for mutations other than forward ones, remains to be seen. Meanwhile, the analysis provides some specific orientation towards the general problem and emphasises the need for much more

research, both for genetics *per se* and for radiation hygiene, which relies heavily on the 7-locus test as a data base for setting standards.

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Culture of human malaria parasites *Plasmodium falciparum*

MALARIA is a major cause of morbidity and mortality, claiming an estimated one million lives a year in Africa alone¹. *Plasmodium falciparum*, the most important agent of human malaria, has not been maintained for more than 2–6 d *in vitro*^{2–10}. The development of a vaccine for malaria depends on suitable culture methods for the production of relevant antigens¹. We describe here a new culture method for *P. falciparum*, which involves an inoculum of cryopreserved parasites and make possible an analysis of merozoite-erythrocyte interactions, growth for more than 3 weeks *in vitro* and the collection of merozoites. Merozoites are of particular interest because vaccination with the merozoites of *P. knowlesi* protects rhesus monkeys from infection with this species of malaria parasite¹¹.

Parasites for culture were obtained from a splenectomised chimpanzee (*Pan troglodytes*) inoculated with a chimpanzee-adapted isolate of the Malayan Camp strain of *P. falciparum*¹². Nine days after inoculation 52% of its erythrocytes were parasitised with ring forms and 6% with schizonts. A sample (50 ml) of this infected blood was collected in 500 U of heparin and cryopreserved in 60 aliquots by the method of Higgins *et al.*³.

Growth of *P. falciparum* in erythrocytes from five species was tested. Blood was drawn in heparin from human volunteers, owl monkeys (*Aotus trivirgatus griseimembra*), rhesus monkeys (*Macaca mulatta*), Hartley guinea pigs (*Cavia porcellus*) and also from the chimpanzee which had been infected (and then cured with mefloquine¹³). These erythrocytes were separated from plasma and leukocytes, mixed with an inoculum of thawed parasitised chimpanzee erythrocytes, and the mixture was cultured at a low oxygen tension in modified tissue culture medium 199. Samples were taken at the times indicated, thin smears were made and stained with Giemsa and 1,000 erythrocytes were examined for parasites. A representative experiment is presented in Table 1. Erythrocytes from humans and the chimpanzee, both of which can be infected *in vivo* with the Camp strain of *P. falciparum*, were parasitised *in vitro*. Erythrocytes from rhesus monkeys and guinea pigs, which cannot be infected *in vivo*, were not invaded to a significant extent *in vitro*. Other experiments provided evidence that erythrocytes from owl monkeys (which can be infected *in vivo*) support growth *in vitro*; too few experiments have

Table 1 Malaria grown in four species of erythrocytes

Test erythrocytes	Trophozoites at 24 h	Trophozoites at 72 h	Ratio of 72 h to 24 h
Human	3.7±1.5	23.0±3.1	6.2
Chimpanzee	5.3±0.9	33.3±1.7	6.3
Rhesus	3.0±1.2	1.3±0.3	0.4
Guinea pig	5.5±2.5	1.8±0.5	0.3

Numbers of trophozoites are given per 1,000 erythrocytes (mean ± s.e. of triplicate cultures).

Test erythrocytes and thawed³ chimpanzee erythrocytes (50% parasitised with ring forms; schizonts did not survive thawing) were mixed at a ratio of 25:1 and cultured at a final concentration of 5×10^7 per ml in an atmosphere of 3% CO₂, 6.6% O₂, balance nitrogen at 37 °C (3 ml per 5-cm² flask, or 0.2 ml per microtitre well). Culture medium 199 (with Earle's modified salts) was enriched with glucose (2 mg ml⁻¹), 2 mM glutamine, 3×10^{-5} M 2-mercaptoethanol, (±)-α-tocopherol (emulsified) (30 µg ml⁻¹), gentamycin (25 µg ml⁻¹), 10% heat-inactivated foetal bovine serum, and 10 mM N-Tris-(hydroxymethyl)methyl-2-aminoethanesulphonate (TES) buffer. (One part TES acid to 1.4 parts TES sodium salt gives a pH of 7.35 at 37 °C). Leukocytes and plasma had been removed from the erythrocytes by passage through a column of cellulose powder¹⁴ followed by washing the cells several times in media.

been done to comment on the finding⁹ that they support growth of *P. falciparum* less well than do human erythrocytes.

In these short term cultures the parasites grow first in the erythrocytes of the chimpanzee inoculum (ring forms predominated at 0 h, more mature trophozoites at 24 h and schizonts at 42 h); then at 48 h merozoites invaded new erythrocytes (young ring forms were also seen at this time in the new erythrocytes). By 72 h the parasite had grown to the trophozoite stage again. At both 48 and 72 h the cultures with human erythrocytes had 15 to 50 times more parasites than cultures with rhesus erythrocytes. Small numbers of degenerating parasites were seen in all cultures. The viability of the cryopreserved parasite inoculum varied from 20 to 50% as judged by the numbers of maturing trophozoites after 24 h of culture.

Long term culture was also possible. Schizonts were concentrated by centrifugation every 2 or 4 d and reinoculated into a culture with new human erythrocytes. The parasite exhibited some asynchrony of growth: on day 22 of culture

Fig. 1 Malaria parasites after 22 d in culture. *a*, Merozoites invading erythrocytes (top right), schizonts (top left and middle), trophozoites (bottom middle); *b*, ring forms; *c*, a gametocyte. Parasites were cultured essentially as described in Table 1, except that every 2 or 4 d the erythrocytes were centrifuged at 400g, resuspended in a small volume of medium, centrifuged in capillary tubes at 1,300g for 2 min, and the capillaries were scored and broken to collect the upper brown layer of schizonts (usually 3×10^7 cells) which was added to a new 15-ml subculture containing 3×10^8 human erythrocytes in a 22-cm² flask. The medium was usually changed every 48 h with the subculture. After the first 4 d of culture, an extra 15 mM TES buffer was added, and 20% autologous human serum replaced the foetal bovine serum in the medium.

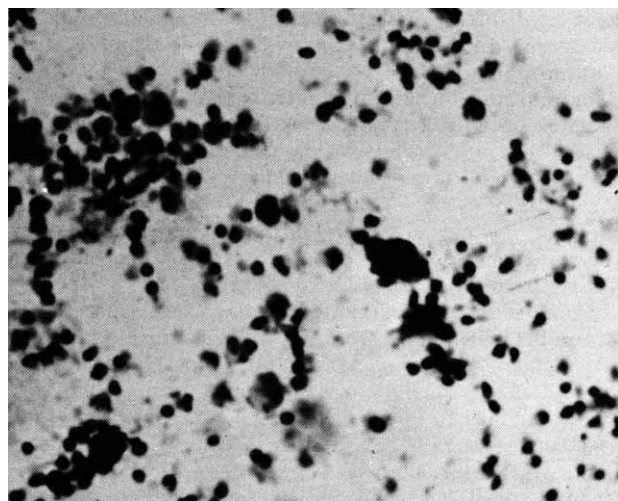
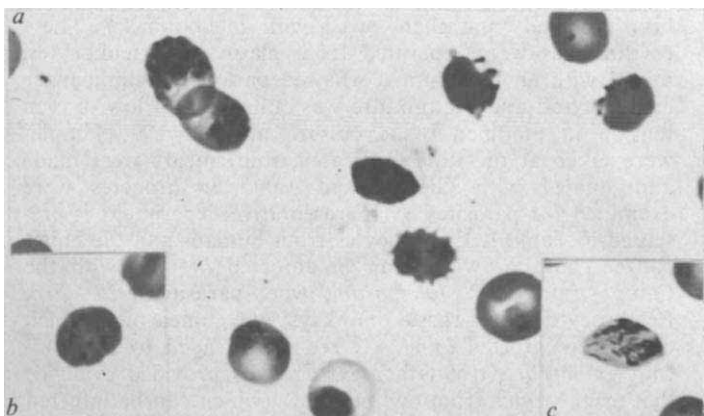


Fig. 2 Thawed parasitised chimpanzee erythrocytes (90% parasitised after a centrifugation step in capillary tubes) were cultured without added erythrocytes, but otherwise essentially as described in Table 1. After 44 h, the culture was centrifuged at 400g for 8 min. The supernatant was then taken and centrifuged at 1,000g for 10 min and the small grey pellet was resuspended, smeared and stained with Giemsa.

schizonts, invading merozoites, ring forms, trophozoites and what appeared to be immature gametocytes were seen (Fig. 1). The total number of schizonts recovered from each subculture was approximately equal to the number added 2 d previously, except on day 22 when twice as many schizonts were recovered. This increased yield followed an increase in the frequency of changing the medium from every other day to every day. A significant metabolic production of acid after 1 d in culture was indicated by the yellow colour of the phenol red in the medium. The addition of a flow system to continually replenish the medium should result in better yields. Further manipulation of culture conditions might also result in the production of mature gametocytes which could be used to study the sexual cycle *in vitro*.

Merozoites were obtained by culturing parasitised erythrocytes to the schizont stage, centrifuging at low speed to remove the unruptured schizonts, and then centrifuging at high speed to pellet the free merozoites (Fig. 2). These *P. falciparum* merozoites have not yet been examined for viability, but are expected to have a lifespan measured in minutes¹⁵.

Experiments to evaluate the importance of the various components of the culture medium are incomplete. Others have reported improved growth of malaria parasites with the use of TES as a buffer⁷, the removal of leukocytes¹⁶ and the use of a low oxygen tension^{17,18}.

As expected from work with *P. knowlesi*¹⁹⁻²¹, the growth of *P. falciparum* in various mammalian erythrocytes *in vitro* correlated with the susceptibility of the erythrocyte donors *in vivo*. This erythrocyte specificity apparently resides in the interactions between the erythrocyte and merozoite surfaces, since very few merozoites adherent to erythrocytes were noted at 48 h in cultures containing erythrocytes from non-susceptible species. The few interactions that were noted probably occurred with residual chimpanzee erythrocytes from the inoculum. This culture system is being used to study *P. falciparum* interactions with various human blood group erythrocytes with and without enzyme treatments (L. H. Miller *et al.*, manuscript in preparation), as reported for *P. knowlesi*²⁰⁻²³.

Further improvements in the culture of *P. falciparum*, in particular the production of large numbers of merozoites, should facilitate biochemical and immunological studies that may lead to an effective vaccine for malaria.

While this manuscript was in preparation, Trager and

Jensen reported the continuous culture of *P. falciparum*²⁴.

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Direct estimation of frequency of cytotoxic T lymphocytes by a modified plaque assay

THYMUS-DERIVED lymphocytes (T) have been shown to have a major role in cell-mediated immunity against foreign transplants and syngeneic tumours^{1,2}. *In vitro*, several assays have been developed to measure thymus-dependent cell-mediated immunity, making use of the cytotoxic potential of these effector cells³. Precise estimates of the number of cytotoxic T lymphocytes (CTL), however, are not possible by available methods. We report here a new method to quantify the CTL present in a mixed cell population. The method is based on the ability of CTL to kill specifically adjacent target cells fixed on monolayers and to form zones of lysis or plaques.

Monolayers of target cells were prepared on poly-L-lysine-coated Falcon plastic Petri dishes as described before⁴. The effector lymphocytes were generated after either *in vivo* sensitisation or *in vitro* sensitisation against tumour allografts. Known numbers of effector lymphocytes were added on the target monolayers and the plates were incubated at 37 °C in an atmosphere of 5% CO₂. After incubation plates were washed with minimum Eagle's medium (MEM), dead tumour cells were stained with eosin, and the plates were washed again with MEM. Zones of dead target cells or plaques were counted under the microscope. A cluster of more than four dead target cells was considered a plaque (Fig. 1).

Several representative experiments are shown in Table 1. Both the *in vivo* and *in vitro* sensitised alloimmune lymphocytes (BALB/c anti-EL-4) form plaques on EL-4 target-cell

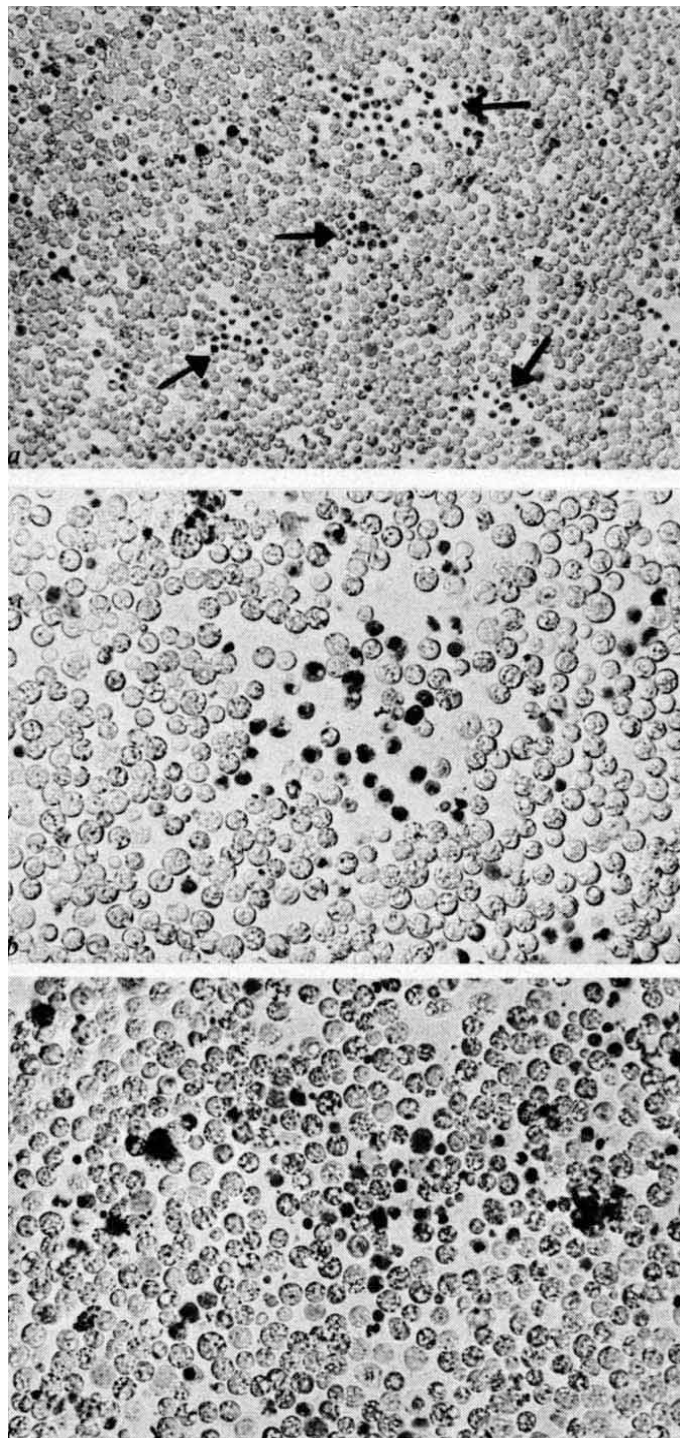


Fig. 1 A monolayer prepared with EL-4 tumour cells and plaques developed by *in vitro*-generated anti-EL-4 cytotoxic lymphocytes. a, Arrows point to plaques ($\times 125$); b, a plaque ($\times 250$); c, a monolayer background lacking plaques ($\times 250$).

monolayers. The specificity of plaque formation was indicated by the absence of plaques when unrelated target-cell monolayers (G-35) were used. Furthermore, normal lymphocytes or lymphocytes cultured alone did not form plaques. A linear relationship was usually obtained between the number of effector cells plated and the number of plaques observed, indicating that each plaque was presumably formed by one CTL. As expected, the *in vitro*-generated secondary response yielded many more plaques when compared with the *in vivo* immune response (experiments 1 and 4, Table 1). Treatment of effector lymphocytes with specific anti-thymocyte serum and complement abrogated completely

both the formation of plaques and the cell-mediated cytotoxic activity obtained by the ^{51}Cr -release assay (experiment 5, Table 1). Neither serum nor complement alone had an adverse effect on plaque formation. These results demonstrated that alloimmune lymphocytes form specific plaques on corresponding target cell monolayers and that the effector cells are thymus-derived lymphocytes.

The relationship between plaque formation and the release of ^{51}Cr from target cells as measures of cell-mediated cytotoxicity was investigated. In all cases, when effector

populations produced plaques, they also showed cytotoxicity by ^{51}Cr release (Table 2). In several experiments, effector cells with strong cytotoxic activity yielded a large number of cytolytic plaques. That the same population of effector cells exerted these two properties, is corroborated by the findings that reduction of cytotoxic activity by absorption on specific target cell monolayers was paralleled by a marked reduction in the number of plaques (experiment 1, Table 2). Experiments with *in vitro* sensitisation of C57BL/6 splenocytes against syngeneic EL-4 leukaemia cells have

Table 1 Frequency of CTL in alloimmune lymphocyte preparations

Experiment no.	Effector cells		No. of cells plated	Monolayer	Plaque-forming units		%
	Sensitisation	Specificity			Observed	Per 10^6	
1	<i>In vitro</i>	Anti-EL-4	3.1×10^5	EL-4	2,380	7,850	0.79
	<i>In vitro</i>	Anti-EL-4	1.5×10^5	EL-4	935	6,171	0.62
	<i>In vitro</i>	Anti-EL-4	3.1×10^5	G-35	0	0	0
	<i>In vitro</i>	Anti-EL-4	1.5×10^5	G-35	0	0	0
	<i>In vivo</i>	Anti-EL-4 PEC	3.1×10^5	EL-4	80	264	0.03
	<i>In vivo</i>	Anti-EL-4 PEC	1.5×10^5	EL-4	35	245	0.03
	<i>In vivo</i>	Anti-EL-4 PEC	3.1×10^5	G-35	0	0	0
	<i>In vivo</i>	Anti-G-35 PEC	3.1×10^5	G-35	70	210	0.02
	<i>In vitro</i>	Anti-EL-4	3.1×10^5	EL-4	2,800	9,240	0.92
2	<i>In vitro</i>	Anti-EL-4	1.5×10^5	EL-4	1,210	8,680	0.87
	<i>In vitro</i>	Anti-EL-4	7.5×10^4	EL-4	600	8,400	0.84
	Lymphocytes alone		3.1×10^5	EL-4	0	0	0
3	<i>In vitro</i>	Anti-EL-4	3.1×10^5	G-35	0	0	0
	<i>In vitro</i>	Anti-EL-4	3.1×10^5	EL-4	(4,410; 3,780; 4,340)*	13,778	1.38
	<i>In vitro</i>	Anti-EL-4	1.5×10^5	EL-4	(1,990; 2,275; 2,030)	14,555	1.46
4	<i>In vitro</i>	Lymphocytes alone	3.1×10^5	EL-4	0	0	0
	<i>In vivo</i>	Anti-EL-4 PEC	3.1×10^5	EL-4	210	693	0.07
	(2 h)		1.5×10^5	EL-4	85	561	0.06
		Normal PEC	7.5×10^4	EL-4	70	924	0.09
			3.1×10^5	EL-4	0	0	0
	<i>In vivo</i>	Anti-EL-4 PEC	3.1×10^5	EL-4	840	2,772	0.28
			1.5×10^5	EL-4	210	2,793	0.28
		Normal PEC	3.1×10^5	EL-4	0	0	0
	<i>In vitro</i>	Anti-EL-4	6.25×10^5	EL-4	2,100	3,360	0.34
5	<i>In vitro</i>	Anti-EL-4	3.1×10^5	EL-4	1,890	4,515	0.45
	Lymphocytes alone		6.25×10^5	EL-4	0	0	0
	<i>In vitro</i>	Anti-EL-4 +	6.25×10^5	EL-4	0	0	0
		Anti T + C*	3.1×10^5	EL-4	0	0	0
	<i>In vitro</i>	Anti-EL-4	6.2×10^5	G-35	0	0	0
6	<i>In vivo</i>	Anti-EL-4 PEC	2.5×10^6	EL-4	840	336	0.034
	Normal PEC		2.5×10^6	EL-4	0	0	0
	<i>In vivo</i>	Anti-EL-4 PEC	2.5×10^6	C57PM	750	300	0.030
	Normal PEC		2.5×10^6	C57PM	5	2	0

Effector cells (alloimmune lymphocytes) were prepared after *in vivo* and *in vitro* immunisation. Cytotoxic cells were generated *in vivo* by intraperitoneal inoculation of 2×10^7 EL-4 (H-2^b) into BALB/c (H-2^d) mice for anti-EL-4 activity, or 2×10^7 G-35 (H-2^d) syngeneic in BALB/c into C57BL/6 (H-2^b) for anti-G-35 activity. The mice were killed 9–10 d later and the peritoneal fluid exudate was aspirated by a Pasteur pipette after several washes of the cavity. The cells were washed, suspended in MEM at 10^7 per ml and samples of 1 ml were plated on plastic tissue culture dishes. The plates were incubated at 37 °C for 1 h and the non-adherent cells were recovered, counted for viability by Trypan blue dye exclusion and suspended in RPMI or MEM at desired concentrations. Lymphocytes were generated *in vitro* as follows. Mice were primed with 2×10^7 tumour cells and 40–60 d later they were killed and their spleens were removed in sterile conditions. The cells were teased apart in balanced salt solution (BSS), filtered through a nylon screen and washed once. Cells were then treated with Gey's solution to lyse red blood cells, washed twice with BSS and resuspended to 10^7 per ml viable cells in RPMI 1640 containing 10% foetal calf serum (FCS). The stimulating tumour cells were prepared as above for responding cells and treated with 30 μg mitomycin C per 10^7 cells. The cells were incubated at 37 °C for 40 min and washed four times with BSS and resuspended to 5×10^6 per ml viable cell in RPMI 1640 containing 10% FCS. Sensitisation was done in 50-ml Falcon conical tubes in which 3 ml of the responder cells (30×10^6 per ml) and 1 ml of stimulator (5×10^6 cells) were mixed and the total volume was brought up to 10 ml. The tubes were incubated for 3–5 d at 37 °C in a 5% CO_2 incubator. At the end of incubation, dead cells were removed using Ficoll-Isopaque. The cell preparation was then brought to the desired concentration in RPMI 1640 + 10% FCS and used for plaque assays or for ^{51}Cr -release cell-mediated cytotoxicity. Monolayers were prepared on 35 $\times$ 10-mm Falcon plastic dishes (no. 3001) coated with poly-L-lysine. One ml of poly-L-lysine (50 μg ml⁻¹, molecular weight 85,000, Sigma, Mi) in phosphate-buffered saline (PBS) (Dulbecco's PBS + CaCl_2 + MgCl_2) was added to each plate and left at room temperature overnight. Plates were washed with PBS before use. Target cells used to prepare the monolayers were either tumour cells or peritoneal macrophages (PM). Tumour cells (EL-4 and G-35) were maintained in ascites form *in vivo*. The ascites fluid containing tumour cells was washed and tumour cells were suspended in PBS at 10^7 per ml. Target cells (1 ml) were added to each plate, which were then left at room temperature for 10 min, centrifuged at 200g for 5 min at 20 °C and washed three or four times with PBS. In most experiments, 1 ml of 0.2% Trypan blue in saline was added to each plate, left at room temperature for 3–5 min, washed with PBS, and monolayers were examined for viability under the microscope. PMs were derived from mice inoculated with 3 ml of thioglycollate 3 d before collection. Peritoneal cells were prepared as described above for tumour cells. Plaque-forming units were estimated as follows. Effector lymphocytes were added at desired concentrations in a total of 1 ml per plate. Plates were incubated at 37 °C in an atmosphere 5% CO_2 for 2–4 h. Plates were washed with 2 ml MEM, and stained with 1 ml of 1% eosin dye for 3–5 min. The plates were washed with PBS and examined under the microscope. In several experiments, monolayers were fixed by treating the plate with 0.2% formaldehyde overnight at 5 °C and then washed with PBS. Plaques were scored under the microscope. Manual interplay with the condenser facilitated location of plaques. In general, the plates were placed on a microscope slide with a square drawn calibrated for 1/5 the surface area of the Petri dish. The total number of plaques per square was routinely counted and the number of plaques per 10^6 lymphocytes was thus estimated.

*In this experiment each effector cell concentration was tested in triplicate.

†Treatment with specific anti-thymocyte serum and C' (ref. 11) was as follows: 0.3 ml of 1:10 dilution of specific rabbit anti-thymocyte serum and 0.3 ml of 1:10 normal rabbit serum (absorbed with tumour cells) as source of complement was added to 10^6 lymphocytes; the mixture was incubated at 37 °C for 45 min and the cells were washed with MEM and resuspended at the original concentration.

Table 2 Correlation between plaque formation and cell-mediated cytotoxicity

Experiment no.	Effector cells	No. of cells plated	Monolayer	Plaque-forming units Observed	%	% CMC to ⁵¹ Cr EL-4
1	BALB/c anti-EL-4	1 × 10 ⁵	EL-4	1,650	1.65	97.0
	BALB/c anti-EL-4 absorbed on EL-4 monolayer*	1 × 10 ⁵	EL-4	225	0.22 (87)†	49.1 (49)
	BALB/c anti-EL-4 absorbed on G-35 monolayer	1 × 10 ⁵	EL-4	1,540	1.54	83.6
2	BALB/c anti-EL-4	1 × 10 ⁵	G-35	0	0	0
	BALB/c anti-EL-4	1 × 10 ⁵	EL-4	420	0.42	58.7
	Normal BALB/c spleen	5 × 10 ⁵	EL-4	15	0.003	0

Effector cells were generated *in vitro* in one way mixed lymphocyte-tumour cultures. 5 × 10⁶ BALB/c splenocytes were mixed with 2.5 × 10⁵ mitomycin C (50 µg ml⁻¹)-treated EL-4 cells in tubes (125 × 16 mm; Falcon no. 3033) in RPMI culture medium supplemented with 10% foetal calf serum. Six days later cultures were collected and the cells were separated on Ficoll-Hypaque to remove dead cells. Cell-mediated cytotoxicity (CMC) with ⁵¹Cr target cells was assayed as described previously⁴ at an effector-target cell ratio of 10:1.

*Absorption on monolayers was done as described before⁴.

†Numbers in parentheses represent percentage reduction.

produced 5–10 times less plaque-forming units (0.04–0.12%) than allogeneic sensitisation. The ⁵¹Cr release in the syngeneic sensitisation was weaker than the allogeneic system.

Several parameters have been examined in an effort to generalise the applicability of the CTL plaque assay and also to optimise the sensitivity of the assay. For example, we found that monolayers can be prepared with target cells other than tumour cells, such as thymocytes or thioglycolate-induced peritoneal macrophages (experiment 6, Table 1). The plates containing the plaques can be fixed with 0.2% formaldehyde and kept at 4 °C without any significant change in the number of plaques. Spontaneous lysis of target cells on the monolayer interferes with plaque reading. Plaques were assayed only when the monolayer target cell showed >95% viability. To distinguish between dead cells present at the initiation of incubation from those which occurred after incubation, a double staining technique was used. The target cell monolayers were first stained with Trypan blue and after plating of the lymphocytes, the monolayers were stained with eosin. The time of incubation of the assay varied from 2 to 5 h depending on the source of effector cells used. *In vitro* sensitised cells required less than 2 h, whereas the *in vivo* sensitised lymphocytes required 4 h (experiment 4, Table 1). Longer incubation periods resulted in a mixture of clear and stained plaques, and these were difficult to quantify and reproduce.

The percentage of CTL in mixed alloimmune preparations as obtained by the plaque assay ranged from 0.05 to 2% of the total plated on the monolayer. These percentages may be underestimates due to the limitations of the assay conditions. For example, (1) CTL may be heterogeneous in their state of differentiation and may express different degrees of cytotoxic activity. In such cases, only CTL that can kill more than four target cells will be scored while CTL which will kill less than four target cells will be missed in our assay. (2) The plating efficiency is not high, that is, CTL with high affinity to target cells may be preferentially selected, and (3) lysis of target cells may not be revealed by dye-exclusion techniques.

Although our plaque assay measured directly the number of CTL, indirect methods have been reported which estimate the number of CTL by their ability to bind to target cells and form conjugates. For example, with alloimmune peritoneal exudate cells (PEC), the number of CTL were estimated to be >6% by Martz⁵ and up to 35% by Berke *et al.*⁶

It is difficult to ascertain from our studies the precise number of precursors of CTL present in a non-immune lymphoid cell population. Such numbers, however, have been reported for precursors which mediate mixed lymphocyte culture and graft *versus* host reactions. It was reported that 1–3% responsive cells became stimulated to proliferate in the mixed lymphocyte interaction⁷, 1–2% induced local graft *versus* host reaction⁸, 6% of parental T cells bear surface

receptors which share idiotypic determinants with IgG fractions of alloantibody specific for a particular major histocompatibility complex (MHC) haplotype⁹, and 4.5–6% participate in a systemic graft *versus* host reaction in the rat¹⁰. If CTLs arise by expansion of the precursor pool after antigenic stimulation then the percentage of uncommitted precursor CTL for one MHC will be less than 1%. This percentage agrees with that postulated by Ford *et al.*¹⁰

In conclusion, the plaque assay described here was shown to enumerate specific cytotoxic T lymphocytes present in a mixed cell population. This modified plaque assay should be helpful in elucidating several aspects of cell-mediated immunity such as cytotoxic potential, affinity, heterogeneity, specificity, and morphological characteristics of effector T lymphocytes once isolated.

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In vitro and *in vivo* radiosensitivity of human tumour cells obtained from a pancreatic carcinoma xenograft

HUMAN tumours show a wide range of clinical response to radiotherapy, but the extent to which this reflects differences in intrinsic cellular radiosensitivity, extent of tumour cell hypoxia, or host reaction against the tumour is unknown. For tumours in laboratory animals, various assays for clonogenic cells are available. These have shown that the range

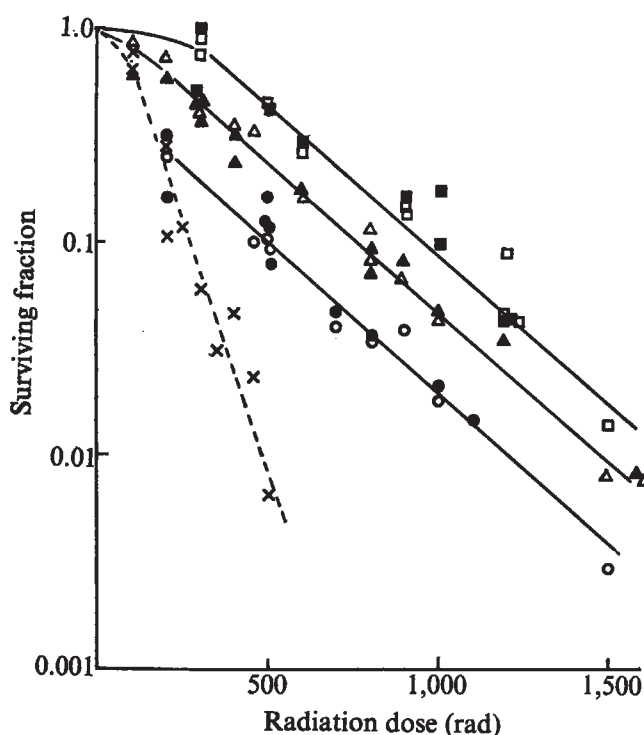
of radiosensitivity of aerobic cells is quite narrow^{1,2} and that the hypoxic fraction of clonogenic cells within tumours of palpable dimensions is often in the range 10–35%. Until now the lack of satisfactory assay for clonogenic cells has prevented similar studies on human solid tumours treated *in vivo*. We have developed two assays for clonogenic cells from a human metastatic pancreatic carcinoma propagated as a xenograft in immune-suppressed CBA/lac mice. This xenograft was one of a series established by Pickard *et al.*³

In the first assay, tumour-cell suspensions were incubated in Ham's medium with the addition of 15% calf serum, 0.3% agar and rat red blood cells in 17-mm diameter test tubes using a double layer technique. Colonies of 50 cells or more were counted at 28 d and the plating efficiency was 20–40%. In the second assay, tumour-cell suspensions in medium, serum and agar were grown in diffusion chambers implanted into the peritoneal cavity of C57BL mice, previously treated with whole-body radiation. Colonies, growing slightly more quickly than in the first assay, were counted at about 18 d and the plating efficiency was 10–20%. Detailed descriptions of the experimental methods are being prepared for publication. Chromosomal analysis of the cells after 16 d growth in culture verified a human karyotype, with aneuploidy ranging from 42 to 68 chromosomes and a mode of 62.

Tumours measuring 5–8 mm in diameter were grown in the leg muscles of immune-suppressed mice and were irradiated *in vivo* using ⁶⁰Co γ rays. The animals were killed and the tumours removed either immediately after irradiation or 18 h later, and cell suspensions were prepared for assay. Acute hypoxia was induced by killing mice by nitrogen asphyxiation 15 min before the start of irradiation. *In vitro* aerobic irradiation was carried out on freshly prepared tumour-cell suspensions.

The results of *in vitro* irradiation and *in vivo* irradiation

Fig. 1 Survival curves for the xenografted tumour cells treated with single doses of γ radiation. $\times$, Cells irradiated as a single-cell suspension in aerobic conditions *in vitro* and assayed using the *in vitro* colony assay. The other data are for tumours irradiated *in situ*: $\circ$, $\bullet$, assay immediately after irradiation in air-breathing mice; $\triangle$, $\blacktriangle$, assay 18 h after irradiation in air-breathing mice; $\square$, $\blacksquare$, assay immediately after irradiation in nitrogen-asphyxiated mice. Open symbols indicate the results of the diffusion chamber assay; closed symbols the results of the *in vitro* assay.



in air-breathing and hypoxic conditions are shown in Fig. 1. The two assays agree well and we are unable to detect any systematic differences between them. The *in vitro* cell survival curve has a D_0 of 96 ± 9 rad. (D_0 is the dose that reduces survival by 63% on the exponential part of the curve.) The *in vivo* air-breathing survival curve for animals killed immediately after irradiation shows an initial sensitive component and a radioresistant component, the D_0 of which is 305 ± 16 rad. The D_0 values for the terminal parts of the other two curves are not significantly different from this and we have therefore drawn the three curves parallel. From the vertical displacement of the hypoxic curve from the lowest air-breathing curve we calculate an hypoxic fraction of 0.25. The oxygen enhancement ratio is 3.1 ± 0.4 . The data obtained on tumours that were taken 18 h after irradiation, are displaced vertically from the other air-breathing curve by a factor of 2.2. This displacement shows that the fractional survival of tumour cells obtained at 18 h after irradiation was greater than when the cells were removed immediately. Two mechanisms may be involved: the repair of potentially lethal damage or the selective death or disappearance during the 18-h period of lethally damaged cells. Repair of potentially lethal damage has been reported in cell populations irradiated *in vitro* and *in vivo*^{4,5}. The present data suggest that the 18-h curve is parallel to the other two curves down to radiation doses as low as 200 rad, but more data are required to confirm this.

Limited clinical experience with irradiation in pancreatic carcinoma suggests that this is not usually a radioresponsive tumour⁶. It is therefore of interest that the D_0 values which we have obtained are within the range of values determined using tumours of laboratory animals. The hypoxic fraction is also no less than has been found in experimental tumours, but the significance of this must be interpreted with caution because of the possible influence on this parameter of vascular stroma derived from the mouse. One interpretation of these data, however, is that the response of pancreatic carcinoma to radiotherapy is limited by the high proportion of hypoxic cells. This provides support for current efforts to improve clinical therapeutic response by the use of chemical radiosensitisers or high LET radiation.

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In vivo hyperthermia of Yoshida tumour induces entry of non-proliferating cells into cycle

HYPERTHERMIA (temperatures in excess of 40 °C) is at present receiving widespread interest as a potential method of treating cancer. High temperature can selectively destroy several types of cancer cell *in vitro* and *in vivo*^{1–3}, and extensive lysis has been achieved clinically in limb tumours maintained at 41.5–43.5 °C for 6–8 h (ref. 1). In spite of these encouraging findings, fundamental information is

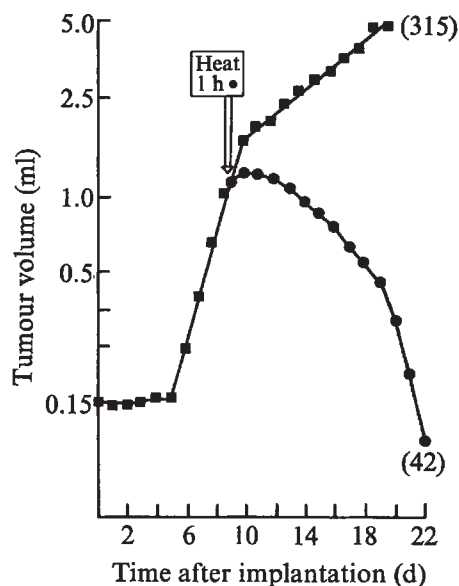


Fig. 1 Growth curve of the Yoshida sarcoma (■), and tumour volume changes after curative hyperthermia (intratumour temperature 42 °C for 1 h) on day 9 after implantation (●). Figures in brackets indicate the numbers of tumours (rats) used to construct the curves.

needed on the factors governing tumour response to heat before the place of this approach in cancer therapy can be defined. Inadequate heating of the Yoshida rat sarcoma (in terms of temperature and/or time relative to tumour volume) increased tumour metastasis and suggested an influence of population kinetics on the outcome of heating^{4,5}. Work *in vitro* has shown hyperthermic cell killing to be preferentially cycle^{6,7} and phase⁸⁻¹¹ oriented. Implication of cell kinetics in the response to heat is further suggested by work on the rabbit VX2 carcinoma. A single heat application (intratumour temperature 42 °C for 1 h) does not destroy this tumour, whereas three such treatments if applied within the tumour cell generation time are curative¹². This report describes changes in the *in vivo* cell kinetics of the Yoshida sarcoma after curative heating at 42 °C, with emphasis on stimulation of non-proliferating tumour cells into cycle.

Details of the history and maintenance of the solid Yoshida sarcoma are described elsewhere². For this work, the tumour was grown in the dorsum of one hind foot of the rats. After implantation of 0.1 ml homogenate the tumour grew progressively killing the host by metastasis in approximately 45 d. The tumour volume growth curve exhibited three distinct phases (Fig. 1)—an initial lag phase, followed by an exponential phase from day 5 to 10, and finally a slower growing 'tail' phase in keeping with the Gompertz growth function for solid tumours¹³.

Tritiated thymidine (³H-TdR; Radiochemical Centre, Amersham; 1 mCi ml⁻¹; specific activity 21 Ci mmol⁻¹) was diluted for injection with normal saline. ³H-TdR was given intraperitoneally to the rats in the following doses: animals to be killed for the percentage labelled mitoses (PLM) and 1-h (flash) labelling work were given a single 400-μCi injection in 1 ml saline; animals for repeated tumour labelling experiments received 100 μCi ³H-TdR every 6 h, and a further 100 μCi 1 h before killing. Autoradiographs of 4-μm paraffin-embedded hemisections of the tumours were prepared using the 'dipping' technique¹⁴ with Ilford K-2 emulsion. After 14 d exposure time, the autoradiographs were processed with D-19 developer and F-5 fixer, and stained with haematoxylin and eosin. For the PLM curves, 500 anaphases and metaphases were counted 'blind', and for the flash and repeated labelling indices (% labelled cells) a

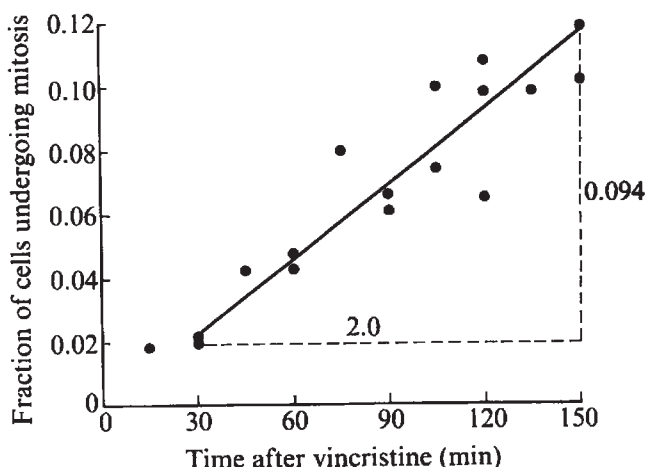
total of 2,000 cells were scored. Vincristine (Oncovin, Eli Lilly, Basingstoke, UK) was injected at a dose of 1 mg kg⁻¹ in 1 ml saline. Paraffin sections (4 μm) were stained as above and the mitotic index (% mitoses) determined by counting 4,000 cells.

The PLM curve of the 1.0–1.5 ml Yoshida sarcoma was constructed from results obtained with 60 tumours examined at 1-h intervals for 30 h. The median cell cycle times shown in Table 1 were obtained by graphical inspection of the curve at the 50% points. The flash-labelling index (*I_L*), or percentage labelled cells 1 h after exposure to ³H-TdR, was 52.5%. These data were used to determine the growth fraction (fraction of the tumour cell population that is actively proliferating, *I_P*; ref. 15), assuming exponential growth and age distribution of the cells. The birth rate (*K_B*), or rate of production of new cells, was estimated by mitotic blocking with vincristine (Fig. 2), birth rate being the slope of the line¹⁶. Tumour doubling time (derived from the slope of the growth curve, Fig. 1) and birth rate data were used to estimate the cell loss factor¹⁷ (or ratio of the rate of cell loss to birth rate).

Tumours of 1.0–1.5 ml were heated at 42 °C (intratumour temperature) for 1 h by immersing the foot bearing the tumour in a water bath. In these conditions, all tumours regressed completely within 14 d (Fig. 1) with cure of the host rats. The effect of hyperthermia on *I_L* is shown in Fig. 3, which provides an indication of the fraction of cells synthesising DNA over the 9 d after heat treatment. For the first 24 h after heat treatment, *I_L* was depressed but recovered to near control levels at 2 d; it then fell progressively to zero by 9 d as the tumour regressed. Changes occurring in the first 48 h after heat treatment were examined in more detail by repeated ³H-TdR-labelling (Fig. 4). In controls repeatedly labelled every 6 h, the labelling index increased to a plateau level of almost 80% labelled cells after 8 h. With curative hyperthermia, a complex sequence of events ensued. Labelling was grossly depressed immediately after heating, but increased rapidly to a maximum of 60% after 8 h; an equally rapid decline in labelling index to a minimum of 5% at 14 h then preceded a final perturbed recovery to a plateau of approximately 60% labelled cells 36 h after heat.

The decline in labelling to 5% at 14 h after heat can only be due to cell death. Thus, more than 90% of the cells proliferating (P cells¹⁸) after hyperthermia had been lost by 14 h, the median cell cycle time of the tumour. This would imply that cells damaged at the time of heating had progressed through one cell cycle and died, possibly in mitosis. It seems unlikely that the rapid recovery of labelling and

Fig. 2 Mitotic accumulation after vincristine arrest in untreated Yoshida sarcoma. Cell birth rate (from slope) = 0.094/2 = 0.047 cells h⁻¹.



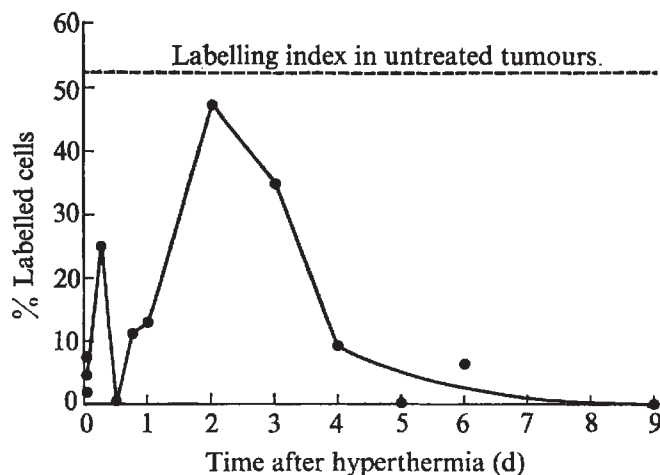


Fig. 3 ^3H -TdR flash-labelling index in the Yoshida sarcoma after curative hyperthermia. From 30 min onwards, each point is the mean of two separate experiments.

mitosis (as determined by vincristine blockade) from 14 to 36 h is caused by cells in cycle at the time of heat treatment. A more likely explanation is the entry into S phase of a population of cells not proliferating (Q cells) at the time of heat treatment; this would repopulate the tumour from 14 h onwards. Further evidence in favour of this is shown in Fig. 4. Repeated labelling was terminated at 14 h after heat, the time of maximum cell death; only a small increase in labelling was subsequently observed. When repeated labelling was commenced at 14 h, the labelling index recovered to the 60% region by 30 h after heat. These two experiments support the hypothesis that the recovery in proliferation from 14 h after heat in this tumour is mainly due to cells in the non-proliferating compartment at the time of heat treatment.

The PLM curve of the tumour 30 h after heat treatment provided durations for the cell cycle components that were not significantly different from those of the untreated tumour (Table 1). This is further substantiation that the changes in cell kinetics produced by curative hyperthermia are primarily in population size and the relationship of the P and Q cell compartments, rather than in cell generation times.

The data imply a preferential destruction of P cells in the Yoshida sarcoma by hyperthermia followed by the

Fig. 4 Repeated ^3H -TdR labelling in untreated Yoshida tumours (Δ), tumours labelled 0–48 h after hyperthermia ($\bullet$), tumours in which labelling was terminated at 14 h after heating ($\square$), or tumours in which labelling was commenced at 14 h and continued to 48 h after heating ($\circ$).

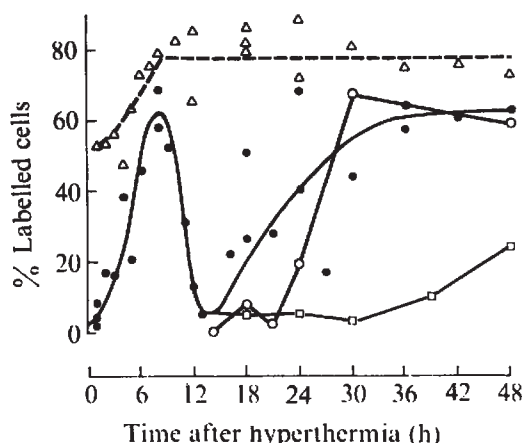


Table 1 Cell kinetic parameters of the Yoshida sarcoma

Tumour volume (ml)	1.0-1.5			
Doubling time (h)	36			
Duration (h) of cell cycle and components:				
T_C	T_S	$T_{G_1}^*$	T_{G_2}	T_M
(14.1)	(9.7)	(—)	(4)	(0.4)
Flash (1 h) labelling index	(I_L)			0.525
Growth fraction	(I_F)			0.678
Birth rate (cells h ⁻¹)	(K_B)			0.047
Cell loss factor	(ϕ)			0.590

*Tumour has no detectable G_1 phase.

recruitment of Q cells into cycle. Similar effects have been observed after radiation¹⁸ or chemotherapy¹⁹, and the timing of recovery has been used to plan fractionated therapy regimens. In spite of apparent kinetic recovery after heat treatment, the tumour regressed within 14 d with complete cure of the animals. The sensitivity of the Yoshida sarcoma to hyperthermia at volumes of 1.0 to 1.5 ml may be due to its high growth fraction ($I_P=0.678$) and the dominant position of the relatively sensitive S phase^{10,11} in its cell cycle (9.7 out of 14.1 h). The failure of cells surviving the first 48 h after treatment to maintain the growth of the tumour implies an inability to repair sublethal damage and/or the operation of host factors in the destruction of the heat-treated tumour. ^3H -TdR-labelled cells and mitoses were observed in tumour sections up to 6 d after heat treatment. This confirms *in vitro* findings²⁰ that several mitoses (up to 10 in the case of the Yoshida tumour) may occur before the expression of lethal hyperthermic damage.

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Antibodies to melanoma cell and BCG antigens in sera from tumour-free individuals and from melanoma patients

THE *Mycobacterium bovis* strain BCG shares antigenic components with several neoplasms, including human malignant melanoma^{1–5}. Other species of bacteria also have antigenic similarities with a guinea pig hepatoma⁶. Antibodies to mycobacteria and other bacterial antigens are universally present in sera from normal humans^{7,8}. Since there is a broad range of shared or cross-reactive antigens among mycobacteria and unrelated microorganisms⁹, it is

conceivable that an immune response by normal humans to mycobacteria has been induced by exposure to taxonomically unrelated microorganisms ubiquitous in the environment. Cytotoxic reactions by lymphoid cells from normal animals and humans to many kinds of tumour cells have been reported¹⁰⁻¹⁵. Antibodies in sera from patients with various neoplasms have often been observed that react with antigens derived from their own tumours or from those of the same histological type¹⁶⁻¹⁸. Humoral responses to tumour-associated antigens have also been noted in sera from normal humans, gibbons and mice¹⁹⁻²¹. We have investigated whether sera from normal, tumour-free humans contain antibodies which can react with melanoma-associated as well as with BCG antigens. If this were the case, perhaps immunological reactions by normal humans to certain tumours are widespread and reflect previous sensitisation by microorganisms.

BCG (Glaxo) was obtained from the US-Japan Cooperative Medical Science Program. Subcultures of BCG were grown, killed by heat, washed and disrupted by sonication. After centrifugation the resulting supernatant was separated and is referred to as BCG-SS (ref. 3).

Melanoma cells were grown in tissue culture and were provided by Dr G. Moore, Denver General Hospital⁴. They originated from a biopsy of a metastatic inguinal node of a male, aged 43 yr, whose primary lesion was on the heel of his right foot. They were treated with 3 M KCl as described before^{3,4}. These KCl extracts have been used previously to investigate antigenic relationships between human malignant melanoma cells and BCG. It has been suggested that the antigenic determinants from this melanoma cell line which are shared with BCG are the group-specific cytoplasmic tumour antigens in melanoma cells⁵.

The melanoma cell KCl extract and BCG-SS were labelled with ¹²⁵I using a modified chloramine T method as previously described^{3,4} and are referred to as ¹²⁵I-mel and ¹²⁵I-BCG, respectively. The labelled antigens were diluted in 1:100 normal human serum in borate buffer so that their concentrations ranged from 0.01 to 0.008 μ g N per 0.1 ml and so that the c.p.m. of 0.1 ml of labelled antigens used in the test procedures were 10,000–15,000.

The primary interactions between labelled test antigens and antibodies were analysed by precipitation of ¹²⁵I-labelled antigen-antibody complexes with anti-human IgG (anti-HGG). The amount of anti-HGG required to precipitate all

the HGG in a given test sample was determined as previously described²². Inhibition and absorption experiments were also carried out to evaluate the specificity of the observed reactions. Details of these procedures have been described before^{3,4}.

Sera from 63 patients with malignant melanoma were obtained shortly after surgery and before the patients were started on a chemioimmunotherapy programme. Thirteen patients had stage 1 disease, four had stage 2, 29 had stage 3 and 17 were stage 4. Ages ranged from 21 to 72 yr. There were 41 male and 22 female patients. Control sera were from 50 healthy subjects, most of whom were personnel at the National Jewish Hospital and Research Center (NJHRC). Sera were not obtained from subjects who had been in contact with human tumours in the laboratory. Ages ranged from 25 to 50 yr and there were 20 males and 30 females. Sera were obtained from two patients at the NJHRC who were under treatment for advanced, active tuberculosis.

When sera from all the patients and normal subjects were tested for their capacity to bind ¹²⁵I-BCG and ¹²⁵I-mel there was a wide range in their capacity to bind both radiolabelled antigens (Fig. 1a and b). As a group, sera from patients with melanoma bound ¹²⁵I-BCG to about the same extent as did sera from normal subjects. The capacity of sera from 17 patients with stage 4 disease to bind ¹²⁵I-BCG was somewhat less than that of normal sera. These differences, however, were not significant ($P < 0.2$). When the same sera were reacted with ¹²⁵I-mel, more antigen was bound by normal sera than by sera from the melanoma patients ($P < 0.025$). The overlapping of data between these two groups made it impossible to correlate disease and the amount of antigen bound by individual serum samples. When sera from patients with stage 4 were compared with normals they were found to have significantly lower ($P < 0.005$) binding values. Lower antibody levels to antigens have been shown when circulating antigen, soluble antigen-antibody complexes or anti-idiotypic antibodies are present, and this may be the case in some patients with far advanced melanoma^{18,23-26}.

A series of experiments was then undertaken to evaluate the specificity of the binding data. The capacity of unlabelled BCG-SS to inhibit binding of ¹²⁵I-BCG and ¹²⁵I-mel by selected sera was examined. Sera from 11 melanoma patients and from seven normal subjects were preincubated

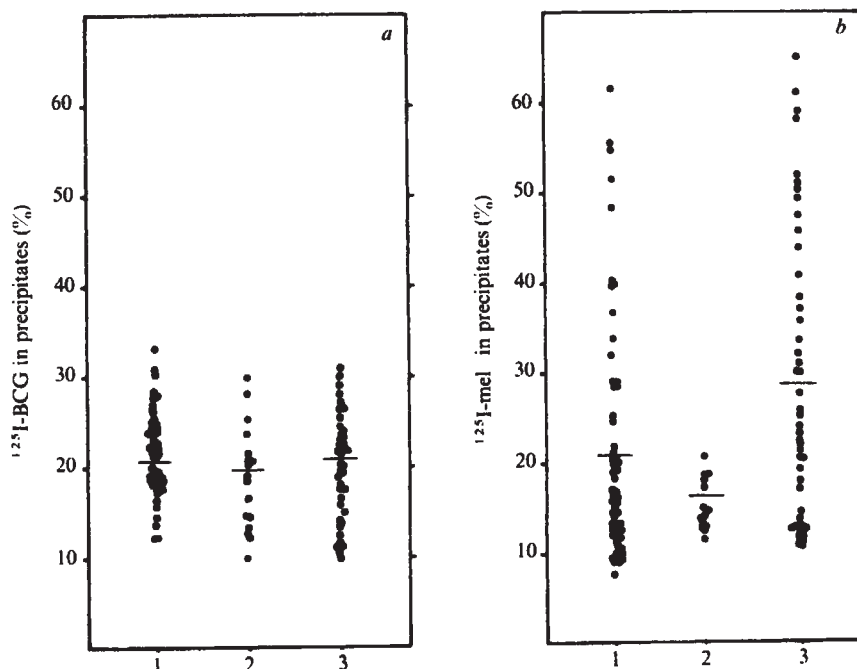


Fig. 1 Results of binding tests using ¹²⁵I-BCG (a) and ¹²⁵I-mel (b) expressed as percentage labelled antigens bound by 0.1 ml of a 1:5 dilution of whole serum. 1, All melanoma (63); 2, Stage 4 melanoma (17); 3, no disease (50).

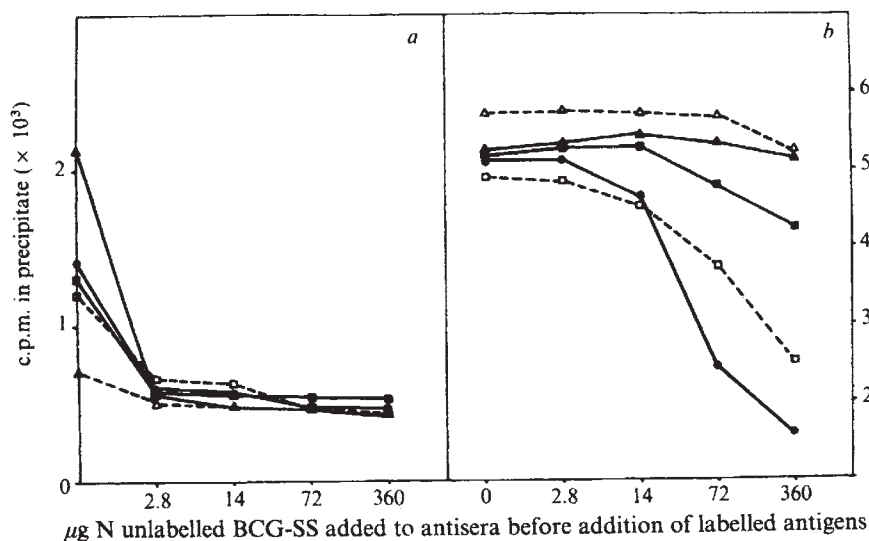


Fig. 2 Inhibition of binding of ^{125}I -BCG (a) and ^{125}I -mel (b) by sera from normal (open symbols) and melanoma (closed symbols) patients after preincubation of sera with dilutions of BCG-SS.

with 300 μg N unlabelled BCG-SS. Control samples were incubated with borate buffer. Twenty-four hours later, ^{125}I -BCG and ^{125}I -mel were added and binding capacities of the antisera were determined. The percentage decrease of binding is shown in Table 1. Unlabelled BCG-SS considerably inhibited the binding as expected to ^{125}I -BCG. To a lesser extent it also reduced the binding by most of these antisera to ^{125}I -mel. This is compatible with observations that some antigenic components are shared between BCG and melanoma cells⁴.

Inhibition studies were carried out in greater detail using sera from three melanoma patients and two normal individuals. Serial dilutions of 0.1 ml of unlabelled BCG-SS were tested for their capacity to inhibit binding of 0.1 ml of ^{125}I -BCG and ^{125}I -mel to 1:5 dilutions of antisera. Controls consisted of 0.1 ml of borate buffer added to the diluted antisera. After overnight incubation, labelled BCG and melanoma antigens were added and binding to these antigens was determined. As little as 2.8 μg N unlabelled BCG-SS inhibited the binding by both normal and melanoma antisera to ^{125}I -BCG, and with increasing amounts there was slightly more inhibition (see Fig. 2a and b). When ^{125}I -mel

was the antigen there was similar inhibition of binding by four of the five sera tested, but more BCG-SS was required. As Fig. 2b shows, 72 μg N caused slight inhibition and 300 μg N were considerably more effective.

Sera from two melanoma patients, two normal subjects and two patients with active tuberculosis were then absorbed with 3×10^7 intact melanoma cells, or with 2×10^8 normal human spleen cells, and then reacted with ^{125}I -mel (Table 2). Sera from patients with tuberculosis were included because they generally have greater capacities to bind ^{125}I -BCG than do sera from normal subjects⁷. After absorption with melanoma cells there was a marked reduction in binding to ^{125}I -mel by all the sera. Absorption of the same sera with normal human spleen cells showed only slight decreases, except for normal serum No. 1, where there was a more moderate decrease in binding. Not shown in Table 1, there was no reduction in binding to ^{125}I -BCG after absorption with 3×10^7 melanoma cells. Unfortunately, greater numbers of melanoma cells were not available for additional absorptions.

Our previous studies and those of others have indicated that antibodies to mycobacteria and various unrelated bacteria and fungi are present in sera from humans with no history of exposure to these microorganisms^{7,8}. In this study antibodies to a melanoma-associated antigen were detected in sera from the normal subjects tested. The binding and inhibition test results indicated that the reactions by normal sera were true immunological manifestations even though the specific stimulating antigen(s) was not identified. Because

Table 1 Percentage decreases of binding of ^{125}I -BCG and ^{125}I -mel by sera from normal subjects and patients with malignant melanoma after preincubation of sera with 300 μg N unlabelled BCG-SS

Antisera from	^{125}I -BCG antigen	^{125}I -mel antigen
Normal humans (No.)		
1	54.5	2.4
2	31.5	7.0
3	70.6	22.8
4	58.2	+3.2
5	29.3	0.4
6	55.5	42.7
7	36.6	19.9
Melanoma patients		
1	29.2	2.9
2	57.2	10.6
3	47.7	0.4
4	40.6	1.2
5	62.7	28.0
6	57.1	23.2
7	48.7	20.4
8	49.1	6.3
9	50.1	32.5
10	50.7	5.9
11	30.7	+1.6

Table 2 Binding of ^{125}I -mel by sera from normal persons, patients with malignant melanoma and tuberculosis after absorption with melanoma and spleen cells

Antisera from	c.p.m. in precipitates		
	Unabsorbed	Absorbed with Melanoma cells*	Normal spleen cells†
Normal human (No.)			
1	7,546	1,827	5,681
2	7,983	1,847	7,742
Melanoma patient			
1	7,408	1,963	7,352
2	7,034	1,762	6,831
Tuberculosis patient			
1	5,373	1,695	5,269
2	1,834	1,520	1,798

* 3×10^7 melanoma cells were used to absorb 1.0 ml of serum.

† 2×10^8 normal human spleen cells were used to absorb 1.0 ml of serum.

$$\text{Percentage decrease} = \left(\frac{\text{c.p.m. in precipitates after preincubation with BCG-SS}}{\text{c.p.m. in precipitates after preincubation with buffer}} - 1 \right) \times 100$$

of the antigenic relationships between BCG and melanoma cells^{2,4,5}, antibodies to melanoma-associated antigens might have been induced by exposure to mycobacteria or other microorganisms. Because the sharing of antigens between tumour cells and bacteria may be widespread, immune responses, whether cellular or humoral, may exist in normal humans to various tumours. Some of the frequently reported and unexplained cellular and humoral reactions to tumour antigens by normal persons¹³⁻¹⁵ may have been induced by exposure to microorganisms. Immune responses by normal subjects¹⁸⁻²¹ or animals to tumour antigens are often referred to as "natural" or "nonspecific". Results of this and previous studies^{2,4,5} imply rather that immune reactions by normal subjects to melanoma-associated antigens can be the result of direct and specific stimulation by antigens of mycobacteria.

It is possible then that just as normal humans respond immunologically to bacterial antigens, they may also respond immunologically to some tumour antigens. If this is the case then immune responses by patients to tumour antigens may sometimes represent an elevation of an "iso-immune" state, rather than a new actively acquired immune response and may influence the course of neoplastic disease.

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effect which was readily reversed by the addition to the medium of small amounts of exogenous PGE₁ (10 ng ml⁻¹) (ref. 5). Corticosteroids have recently been shown to inhibit the biosynthesis of PGE by rheumatoid synovia⁷ and mouse fibrosarcoma HSDM₁ (ref. 8) *in vitro*; the mechanism of this action seems to be by interfering with the release of arachidonic acid from phospholipids⁹. To evaluate further the possible role of PGE in the control of tumour-cell proliferation, we have studied the effects of hydrocortisone and indomethacin on the growth rate of B-16 mouse melanoma *in vitro*. The studies demonstrate that both compounds cause significant stimulation of tumour-cell replication *in vitro*, associated with inhibition of PG biosynthesis, albeit by two different mechanisms. In addition, we have extended the observations on the effects of exogenous PGE₁, by demonstrating that subcutaneous administration of 16,16-dimethyl-PGE₂-methyl ester significantly inhibits tumour growth *in vivo*.

The *in vitro* studies were all performed using B-16 melanoma cultured in modified McCoy's 5A medium, containing 15% foetal calf serum, glutamine (220 µg ml⁻¹), penicillin (100 U ml⁻¹) and streptomycin (0.1 mg ml⁻¹). Cultures were maintained in 75-cm² plastic T-flasks at 37±0.5 °C in humidified 95% air, 5% CO₂, and media were changed on alternate days. Media containing PGE₁, indomethacin and hydrocortisone were sterilised by Millipore filtration. Control media contained identical concentrations of ethanol (< 0.01%). For counting, cells were removed using 0.1% trypsin, EDTA, 1.5 mg ml⁻¹, and mechanical scraping and were resuspended in medium. At least triplicate cell counts were performed using a haemocytometer; cell viability, as determined by vital dye exclusion¹⁰ (Nigrosin 0.2%), ranged from 92.5 to 100% and was not influenced by the addition of exogenous PGE₁, indomethacin, or hydrocortisone. PG concentrations in media and supernatants were measured by radioimmunoassay after organic solvent extraction and silicic acid chromatography as described previously^{5,11}.

In an initial experiment, 2×10⁵ B-16 cells were plated in duplicate flasks containing PGE₁ (1 µg ml⁻¹), indomethacin (10⁻⁸ M), or hydrocortisone (10⁻⁸ M). Exogenous PGE₁ caused a mean 24.4±6.1% inhibition of cell replication (Fig. 1); inhibition was statistically significant at days 4 and 6 (*P* < 0.05 and *P* < 0.001, respectively). As in previous experiments, indomethacin caused a significant (35.5%) inhibition of PG concentrations in the media and resulted in a mean 17.0±3.2% stimulation of cell replication, which was statistically significant at days 4 (+18%; *P* < 0.05%) and 6 (22%; *P* < 0.005%). Hydrocortisone produced similar results, significantly stimulating cell proliferation and decreasing endogenous PGE concentrations in the media.

The relationship between the concentration of exogenous PGE and the degree of inhibition of cell proliferation was tested in duplicate experiments in which 0.5, 1.0, 2.0, 5.0 and 10.0×10⁵ B-16 cells were exposed to PGE₁ (1 µg ml⁻¹) for 48 h. There was a gradual dose-response curve with higher ratios of PGE/cell causing more inhibition (31.4%, 37.5%, 25.8%, 22.8% and 22.4%, respectively).

The effect of hydrocortisone on the proliferation rate was investigated further by plating 2.5×10⁵ cells from one stock culture in duplicate control media as well as duplicate media containing hydrocortisone at 0.3×10⁻⁸ M, 10⁻⁶ M, and 10⁻⁷ M. All three concentrations of hydrocortisone caused significant stimulation of cell replication (Table 1). Maximal stimulation (mean 36.5%) was achieved with 10⁻⁶ M hydrocortisone; comparable data for 0.3×10⁻⁸ M and 10⁻⁷ M were +18.1% and +32.2%, respectively. In spite of the large cell numbers achieved, there seemed to be no contact inhibition of growth. Total PGE biosynthesis (cells+media) was linear in control conditions and was inhibited significantly by hydrocortisone at all three concentrations (Fig. 2). Each of the three concentrations of hydrocortisone inhibited PGE synthesis approximately 50%. The effect of hydrocortisone on PGE concentrations was similar in media and cells, which rules out the possibility that hydro-

Inhibition of tumour growth *in vivo* and *in vitro* by prostaglandin E

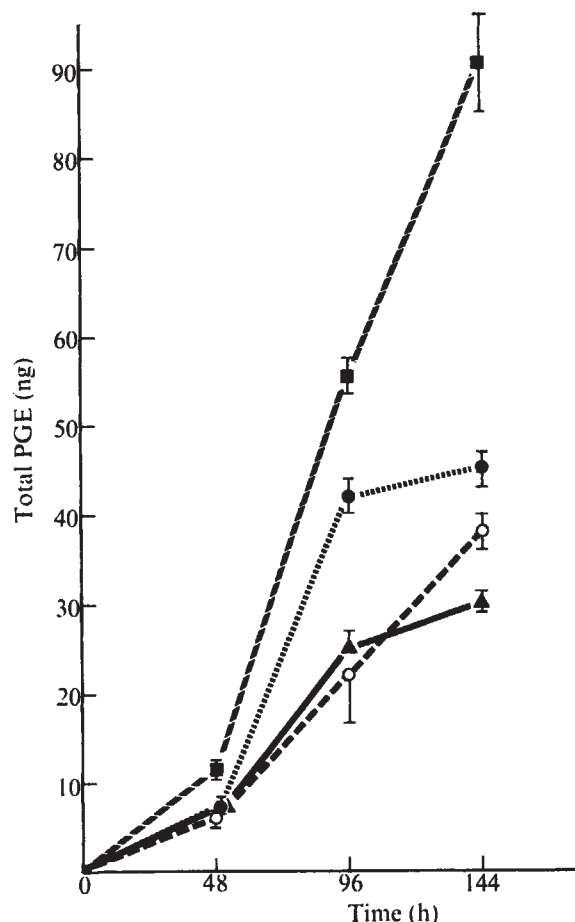
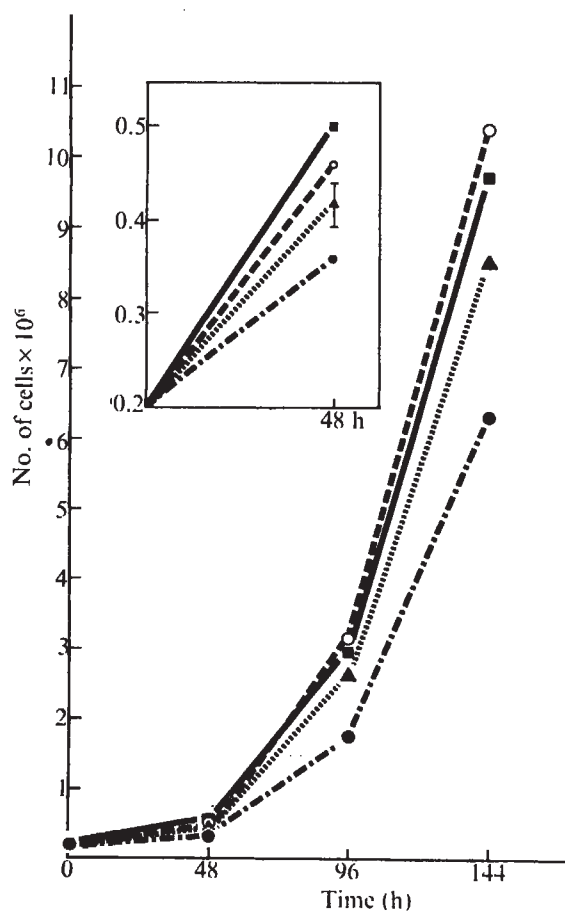
PROSTAGLANDIN E (PGE), both endogenous and exogenous, has been shown to inhibit significantly the rates of tumour-cell proliferation *in vitro*¹⁻⁴. Suppression of PG biosynthesis with indomethacin^{5,6} resulted in stimulation of cell replication, an

Table 1 The effect of hydrocortisone on B-16 proliferation *in vitro*

Hydrocortisone	0 h	Number of cells $\times 10^6$		
		48 h	96 h	144 h
0	0.25	0.40 ± 0.01	4.85 ± 0.55	9.95 ± 0.55
0.3×10^{-6} M	0.25	0.51 ± 0.03	5.80 ± 0.40	10.65 ± 0.75
10^{-6} M	0.25	0.67 ± 0.04	5.97 ± 0.02	11.78 ± 0.35
10^{-7} M	0.25	0.54 ± 0.06	6.55 ± 0.45	12.60 ± 0.90
10^{-8} M	0.25	0.47 ± 0.04	4.65 ± 0.05	9.55 ± 0.65
+ PGE ₁ , 10 ng ml ⁻¹				

The effect of hydrocortisone on B-16 proliferation *in vitro*. Data represent mean $\pm$ s.d. of duplicate determinations at each time point; at least triplicate counts were performed for each determination. Viability exceeded 95% in all cultures. Compared with control values, hydrocortisone caused mean stimulation of cell replication: 0.3×10^{-6} M, 18.1%; 10^{-6} M, 36.5%; 10^{-7} M, 32.5%; 10^{-8} M + 10 ng ml⁻¹ PGE₁, 3.2%.

cortisone only inhibited the release of PGE from cells. Using regression analysis, the degree of stimulation was not significantly related to the degree of inhibition of prostaglandin biosynthesis ($P > 0.05$). Therefore, in order to verify that the effect of hydrocortisone on stimulation of cell replication was, in fact, a result of its induced inhibition of prostaglandin biosynthesis, an identical experiment was carried out in which cells were grown in media containing both 10^{-6} M hydrocortisone and exogenous PGE₁, 10 ng ml⁻¹ (the approximate endogenous concentration in control conditions). Small concentrations of exogenous PGE₁ normalised the growth rate (Table 1).

Fig. 1 Effect of PGE₁, indomethacin and hydrocortisone on B-16 replication *in vitro*. Data represent means of duplicate flasks at each time point; for each determination at least triplicate counts were performed. Δ , Controls; $\blacksquare$, hydrocortisone 10^{-6} M; $\circ$, indomethacin 10^{-8} M; $\bullet$, PGE₁, 1 μ g ml⁻¹.**Fig. 2** Effect of hydrocortisone on PGE biosynthesis. Data represent total (media + intracellular) concentrations mean $\pm$ s.e.m. Each determination was based on duplicate radioimmunoassay determinations on replicate samples. Hydrocortisone: $\blacksquare$, 0 (control); $\circ$, 0.3×10^{-6} M; $\bullet$, 10^{-6} M; Δ , 10^{-7} M. Compared with the controls, hydrocortisone produced significant ($P < 0.05$) inhibition of PGE biosynthesis, 63.0%, 45.1% and 57.7%, respectively. Hydrocortisone produced slightly less inhibition of PGF synthesis; the PGE/PGF rates averaged 3.12 in the control experiments and 2.39 in the hydrocortisone experiments.

In an experiment designed to evaluate if hydrocortisone interfered with the cyclo-oxygenase system, homogenates were prepared in which 5×10^5 sonicated cells were mixed with glutathione ($55 \mu\text{g ml}^{-1}$), hydroquinone ($0.55 \mu\text{g ml}^{-1}$), EDTA ($5.5 \mu\text{g ml}^{-1}$), and arachidonic acid ($500 \mu\text{g ml}^{-1}$) as previously described¹². After 1 h of incubation at 37°C in 95% air and 5% CO_2 , the homogenates were extracted for measurement of PGE concentrations. In the control conditions, homogenates of 5×10^5 cells synthesised 9.7 ng of immunoreactive PGE. Inclusion of hydrocortisone at a final concentration of 10^{-6} M did not alter PGE synthesis (9.6 ng). On the other hand, indomethacin (10^{-8} M) produced 92.9% inhibition of the conversion of arachidonic acid to PGE₂ (0.74 ng).

In *in vivo* studies, 37 3-week old litter mate female C57BL/6J mice (Jackson Laboratories) were injected subcutaneously with 1×10^6 viable B-16 melanoma tumour cells suspended in 0.2 ml of McCoy's medium containing penicillin and streptomycin but no foetal calf serum. They were divided randomly into control ($n = 19$) and test ($n = 18$) groups and within 2 min received a subcutaneous injection in the same area of 0.1 ml of 0.9% sodium chloride containing either 25% absolute ethanol (control group) or an ethanolic solution containing 5 μg of 16,16-dimethyl-PGE₂-methyl ester (test group). The

Table 2 The effect of 16,16-dimethyl-PGE₂-methyl ester on B-16 growth *in vivo*

No.	Tumour weight* (mg)	Control group Cells per g tumour ($\times 10^6$)	Body weight (g)	Total cells in tumour† ($\times 10^6$)	No.	Tumour weight* (mg)	PGE group Cells per g tumour ($\times 10^6$)	Body weight (g)	Total cells in tumour† ($\times 10^6$)
1	—	—	23.0	—	1	—	—	23.5	—
2	230.1	—	20.0	59.46	2	—	—	23.0	—
3	230.8	284.0	19.0	59.64	3	30.6	—	27.0	5.63
4	1,008.7	266.6	20.0	260.65	4	37.0	—	25.5	6.81
5	685.7	—	18.0	177.00	5	—	—	9.0	—
6	834.9	236.8	11.0	215.74	6	183.0	—	15.0	33.70
7	428.0	—	12.2	110.59	7	63.1	—	19.7	11.62
8	477.3	230.8	11.3	123.33	8	481.6	206.3	18.5	88.71
9	487.1	—	14.9	125.86	9	455.0	160.7	15.9	83.81
10	125.9	—	14.5	32.53	10	45.6	—	16.9	8.39
11	465.7	—	13.8	120.33	11	151.7	185.8	11.9	27.94
12	508.0	—	12.0	131.26	12	24.3	—	15.5	4.47
13	492.3	—	14.0	127.21	13	—	—	20.1	—
14	243.7	—	13.0	62.97	14	30.1	—	21.3	5.54
15	432.5	—	11.0	111.75	15	—	—	15.4	—
16	818.5	—	14.8	211.50	16	64.1	—	16.7	11.80
17	276.9	—	17.8	71.55	17	179.7	—	18.8	93.10
18	988.6	273.8	14.5	255.45	18	231.1	—	17.9	42.57
19	136.3	—	19.2	35.22					
Mean (18)	492.8	258.4	15.4	127.3	(13)	152.0	184.2	18.08	28.0
±s.e.	±64.9	±10.4	±0.8	±16.7		±43.4	±13.1	±1.1	±8.0
Mean (19)	466.9	—	—	120.6	(18)	109.8	—	—	20.2
±s.e.	±66.6	—	—	±17.2		±35.1	—	—	±6.5

The effect of 16,16-dimethyl-PGE₂-methyl-ester on B-16 growth *in vivo*.

*Tumour weights represent wet weights immediately after collection.

†Total cells per tumour was determined by tumour weights $\times$ mean cell number per g of tumour in each group.

identical injection procedure was repeated daily. Tumours were apparent approximately 10 d after inoculation in both groups. After 18 d the mice were killed with ether and the tumours were collected and weighed. No necrosis was apparent in any tumour in either group. Tumour cells were separated by passage through a 120 mesh stainless steel grid. After washing twice with medium, the cells were resuspended and counted as described previously. As shown in Table 3, 18 of 19 (95%) mice in the control group developed subcutaneous tumours which weighed an average of 492.8 ± 64.9 mg. In contrast, only 13 of 18 (72%) of the mice in the test (16,16-dimethyl-PGE₂-methyl ester) group developed identifiable tumours. Using a χ^2 test, this difference was statistically significant with a P value of < 0.05 . Furthermore, among the 13 tumour-bearing animals, the mean tumour weight was 152.0 ± 43.4 mg representing 69.2% inhibition; compared with the mean weight of the tumour-bearing controls, this difference was also statistically different with a P value of < 0.001 . Based on the mean number of cells per g of tumour in each group, it was possible to plot the fate of the 10^6 injected cells. In the control group, the tumours represented an average of $120.6 \times 10^6 \pm 17.2$ cells per mouse (including the tumour-free animal) whereas in the test group, they represented $20.2 \times 10^6 \pm 6.5$ cells per mouse. This difference (inhibition by 83.2%) was statistically significant ($P < 0.001$). At the completion of the experiment, the control mice weighed an average of 15.4 ± 0.8 g, and the test group 18.1 ± 1.1 g ($P < 0.05$); there was no correlation between tumour weight and the body weight of the mice.

These studies have further implicated PGE in the control of tumour cell growth. Inhibition of PGE biosynthesis, either by indomethacin or by hydrocortisone results in stimulation of cell proliferation; the effect of both of these compounds can readily be reversed by the addition of small (subthreshold) amounts of PGE₁. The effect of PGE₁ in inhibiting proliferation is, in general, dose related. These *in vitro* observations are further substantiated by the *in vivo* experiments, in which subcutaneous (and after the appearance of tumours, intra-tumour) administration of a long-acting PG analogue resulted in significant suppression of tumour growth.

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Signal for cell fusion

THE mating of the gametes of *Ulva mutabilis*¹ occurs essentially as described for *Chlamydomonas*^{2,3}. When the gametes are mixed, they cluster and agglutinate with the tips of their flagella. Pairs consisting of a (+) and (−) gamete leave the cluster, the cell bodies are brought together and fusion initiated. In *Chlamydomonas* agglutination apparently creates a signal which triggers the later events of cell wall lysis, mating structure activation and cell fusion⁴. The gametes of *Ulva* lack a cell wall⁵ and have apparently no elaborate mating structure^{2,5,6}. This note shows that the signal in *Ulva* activates particular areas of the membrane for fusion only for a limited time, and if fusion does not occur then, the ability to fuse is lost permanently.

Figure 1a shows that fusion is finished within 3–4 min both at 10 °C and 22 °C, but that the numbers which fuse are considerably lower at 10 °C than at 22 °C. The data in Figure 1b indicate that the number is constant above 15 °C,

decreases rapidly with decreasing temperature around 10 °C and reaches zero at 0 °C.

The membranes of the two gametes fuse in a region below the flagella and the fused area increases in size as the two gametes merge into each other. The merging is temperature dependent (Fig. 2, insert), and an Arrhenius plot of the initial rate of merging reveals a low activation energy above 15 °C and a higher below 15 °C. The rate most probably depends on the physical state of the membrane, and it is therefore suggested that the membranes change properties around 15 °C. This is also the temperature below which the number of fusers decreases. The decrease can be explained if fusion can take place only for a limited period of time and if the probability for fusion is temperature dependent below 15 °C.

When the two sexes are incubated separately at low temperature for 30–40 min, and mixed after raising the temperature to 22 °C, no reduction in the number of fusers occurs. Consequently the reduction in the ability to fuse develops after the two sexes have been brought together.

Fig. 1 *a*, The percentage of zygotes formed as function of time at 22 °C (●) and 10 °C (+). The two mating types were mixed at time zero. *b*, The final percentage of zygotes as a function of temperature. The percentage at 22 °C is taken as 100. Gametogenesis was induced as described by Nordby and Hoxmark¹². The experiments were performed at a cell density of 10^6 – 10^7 gametes ml⁻¹. At different times after mixing the (—) and (+)-gametes the process was stopped by adding an equal volume of 2.5% glutaraldehyde in 0.2 M cacodylate buffer pH = 8, and 100–400 gametes (*g*) and zygotes (*z*) were counted in the phase contrast microscope. The percentage of fusion is given as $200 z/g + 2z$.

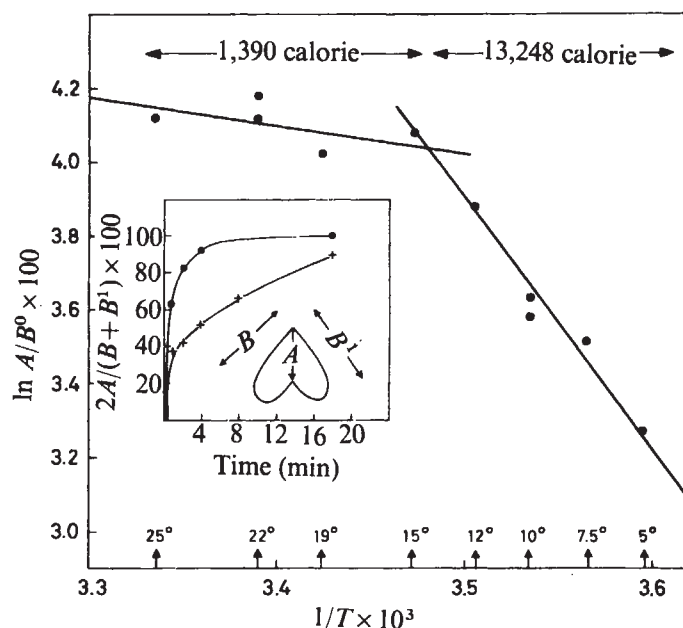
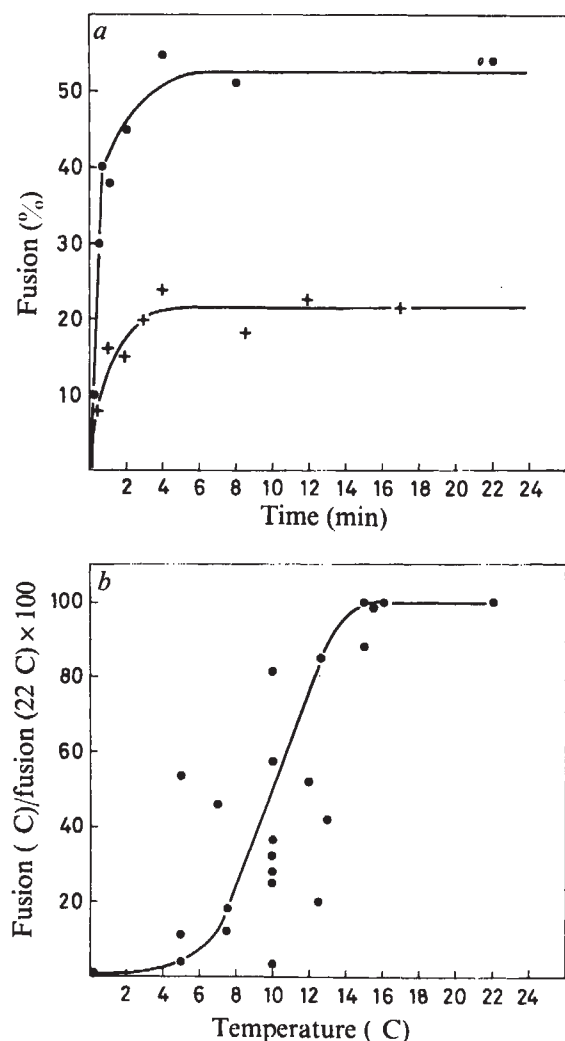


Fig. 2 Relationship between temperature and the rate of merging. The degree of merging is measured by the length of line A (see insert) and is given as the percentage of the average ($n = 25$) length of the fused gametes ($\frac{1}{2}(B+B^0) = B^0$). The insert gives the percentages of merging at different times at 10 °C (+) and 22 °C (●). The percentages obtained 1 min after mixing are used as an approximate measure of the initial rate of merging, and such rates are given in the Arrhenius plot. The lines are fitted to the points by the method of the least squares. Above 15 °C the slope is presumably not significantly different from 0, which means zero activation energy. The zygotes were fixed for 4–5 h (see legend to Fig. 1), filtered on to a Nucleopore filter CPRO 1300 0.8 μ m, dried by the critical point drying method¹³ and coated with Au. The measurements were made directly on the screen of the Jeol ISM-S1 scanning electron microscope ($\times 10,000$), operated at an angle of 0°.

Figure 3 indicates that exposure to 0 °C for only 3–5 min after mixing the gametes reduces the number of fusers to 50%, and exposure for 11 min almost totally prevents fusion.

The effect of cold is simulated at room temperature by cocaine, which is believed to be a membrane stabiliser and prevents virus-induced fusion in mammalian cell strains⁷. When gametes were treated with 5×10^{-3} M cocaine for 15 min, washed three times and mixed immediately, 62% fused. When the gametes were mixed after 5 min and washed after 10 min, the corresponding value was only 2%.

Cocaine at this concentration enables agglutination to occur. Observations also show that at 0 °C the two mating types agglutinate and stay in contact by the tips of their flagella for long periods. It is therefore suggested that this contact starts a process which within minutes makes the gametes incapable of fusion.

To investigate the role of flagellar contact on the fusion process, the flagella were removed with a 15-ml Dounce homogeniser (Pistil B) and the deflagellate gametes mated with biflagellate ones of the opposite sex. The degree of deflagellation could be regulated by the number of strokes, and it appeared that the number of zygotes formed was roughly proportional to the number of biflagellate gametes in the treated sample.

In fixed gametes the flagella stick out from the cell body and their presence is easy to observe. In the zygotes the flagella are directed backwards close to the cell body and it is not always easy to ascertain that all four are present. On inspecting 50 normal zygotes in the phase contrast microscope four flagella were observed in 64%, three flagella in 26%, two flagella in 10% and no zygotes carried one or no flagella. When deflagellated (—) gametes, (no flagella: 73%, one flagellum: 24%, two flagella: 3%) were

Table 1 The effect of con A on pairing

Mating type of the deflagellated gametes	Con A	Mating type of the flagellated gametes	Pairs consisting of: Gametes of the same mating type	Gametes of different mating type	Zygotes	Aggregates
+	+	-	3	70	11	Few
+	-	-	3	78	3	Few
-	+	+	59	0	4	Many
-	-	+	14	51	4	Few

(+) and (-) gametes were deflagellated with six strokes of the Dounce homogeniser (80% gametes with no flagella, 15% with one flagellum, 5% with two flagella) and pretreated with fluorescent con A ($100 \mu\text{g ml}^{-1}$)¹¹, washed four times and then challenged with untreated biflagellate gametes of the opposite sex. The controls were done with untreated deflagellate gametes. Ten minutes after mixing the samples were concentrated in the centrifuge, suspended in 50 μl medium and fixed in 4% formalin. The samples were examined for 10 min each in the fluorescence microscope and the number of pairs and zygotes recorded.

mated with normal (+) gametes, the corresponding numbers ($n=100$) were 7%, 66%, 21%, 3%, 3%. The high occurrence (66%) of zygotes with three flagella in the experiment compared with the control (26%) indicates that a uniflagellate gamete can participate in zygote formation. If the probability of not observing a flagellum which is present is the same in the experiment and the control, the data also suggest that a gamete without any flagella has a very low chance of fusing.

There seemed, however, to be a tendency for these gametes to pair with others without fusion, as indicated by the preponderance of pairs with two flagella in the experiment above. Four per cent of pairs carried four flagella, 22% carried three flagella, 62% carried two flagella, 12% carried one flagellum and 4% had no flagella. In the pairs the individuals adhere to each other just below the flagella where fusion is normally initiated. When both mating types are deflagellated and brought together with repeated centrifugations many pairs but no zygotes are observed.

In *Chlamydomonas*, concanavalin A (con A) causes isoagglutination of one or both sexes depending on the species⁸⁻¹⁰. In *Ulva*, con A (Sigma) isoagglutinates the flagellate gametes of both sexes at a concentration of $25 \mu\text{g ml}^{-1}$ or above, but nevertheless it is possible to reveal a difference between the two kind of gametes. A 50% inhibition of zygote formation is obtained with $4 \mu\text{g ml}^{-1}$ con A. The inhibition is due to con A-specific sites at the (-) gametes, as shown by an experiment in which the gametes were pretreated for 5 min at a concentration of $25 \mu\text{g ml}^{-1}$ con A, washed with medium four times, and mixed with untreated gametes of the opposite sex, which had not been treated with con A but had been washed four times. When the con

A-treated gametes were mating type (+), the percentage of fusion was normal (experiment 53 control 45%). When mating type (-) was pretreated, only 1% fused.

If deflagellate gametes were treated with fluorescein-labelled con A, it could be demonstrated that pairing of (+) and (-) gametes was prevented only by con A treatment of the deflagellate (-) gametes (Table 1). While the flagellate gametes of both sexes are isoagglutinated in the presence of con A, treatment and subsequent washing causes isoagglutination of only the (-) sex. These observations suggest that the region of the membrane which pairs and later fuses, represents a special part which is different in the two kinds of gametes.

In conclusion, the results can be explained if contact between the tips of the flagella creates a signal which activates a particular area of the membrane of each gamete for a limited period of time. If the two areas, which are different, are brought into contact, there is a given probability for fusion. This probability is dependent on the physical state of the membrane, and decreases with temperature below 15°C or if membrane stabilisers like cocaine are added. If fusion does not occur during the defined period, the gametes separate and have lost the ability to fuse for good, either because the membranes are irreversibly deactivated or because a new signal cannot be generated.

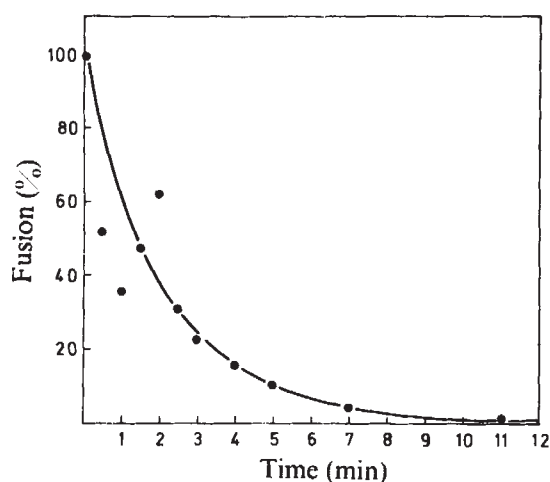
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Fig. 3 The loss of fusion ability with time. The gametes were precooled at 0°C for 25 min, mixed at zero time and at the times indicated 10 μl of the mixture was dropped on to a slide at room temperature and the percentage fusion recorded 15 min later.



Recycling of dissolved plasma membrane components as an explanation of the capping phenomenon

In a recent paper in *Nature*, Bretscher¹ proposed that the capping phenomenon^{2,3} is caused by a continuous, directional flow of the lipid components of the plasma membrane across the cell surface from one end to the other, while the membrane proteins remain dispersed by random thermal motion and therefore do not normally participate. Bretscher's explanation of capping is that the cross linking of membrane proteins by attached antibodies and lectins causes them to be swept along by this lipid

current and collected at the "uroid" portion of the cell surface, where he proposes that the membrane lipid is taken into the cytoplasm (by way of a "molecular filter") for return to the opposite side of the cell as cytoplasmic vesicles. In this way Bretscher can account for the selective accumulation into caps of certain classes of surface antigen, without needing to postulate any communication between the surface and the interior. Cross-linked antigens would simply be swept into caps by the lipid current, while uncross-linked antigens would remain dispersed, just as is actually observed.

As an alternative to Bretscher's theory, I propose that capping results from a directional flow of the entire plasma membrane, including not only its lipid but also its integral protein and carbohydrate components. This alternative hypothesis is an extension of the theory previously proposed by Abercrombie *et al.*⁴ to explain fibroblast locomotion and has been developed primarily on the basis of studies of cell locomotion and particle transport⁵⁻⁷. The basic postulate of my hypothesis is that all the components of the plasma membrane can dissolve into the cytoplasm at least to a slight extent and that in all motile tissue cells true thermodynamic equilibrium exists between the plasma membrane and its components dissolved in the cytoplasm.

According to LeChatelier's principle of mobile equilibria, forces imposed on the reactants of an equilibrium will cause the equilibrium to shift in whichever direction tends to absorb the imposed force⁸. I propose therefore that local stretching of the plasma membrane should shift this equilibrium in favour of the insertion (assembly) of dissolved membrane components into the plasma membrane. Consequently membrane assembly should be concentrated where the plasma membrane is most stretched. Conversely, membrane disassembly, that is, the dissolving of components back into the cytoplasm, should be favoured wherever the membrane is least stretched. In this way, a steady longitudinal pull applied to the plasma membrane by the cytoplasmic microfilaments would result in reassembly of membrane along the leading margin of the cell, followed by rearward flow of this membrane, with disassembly occurring in the more posterior portion of the cell surface. The disassembled (dissolved) membrane components would then be free to diffuse forward through the cytoplasm back to the reassembly area.

Fig. 1 Diagram of the proposed pattern of plasma membrane movement during lymphocyte capping and tissue cell locomotion. Along the leading margin of the cell, plasma membrane components dissolved in the cytoplasm are rapidly intercalated into the surface (membrane reassembly). The plasma membrane flows rearward from this assembly area, exerting traction against any adhering objects and thereby propelling the cell forward. At the rear "uropod" area of the cell, membrane components redissolve in the cytoplasm (membrane disassembly) and diffuse through the cytoplasm back to the front. Bound antibodies, lectins, and so on, are carried to the uropod area by the rearward membrane flow, and being unable to dissolve in the cytoplasm themselves, accumulate there to form caps.

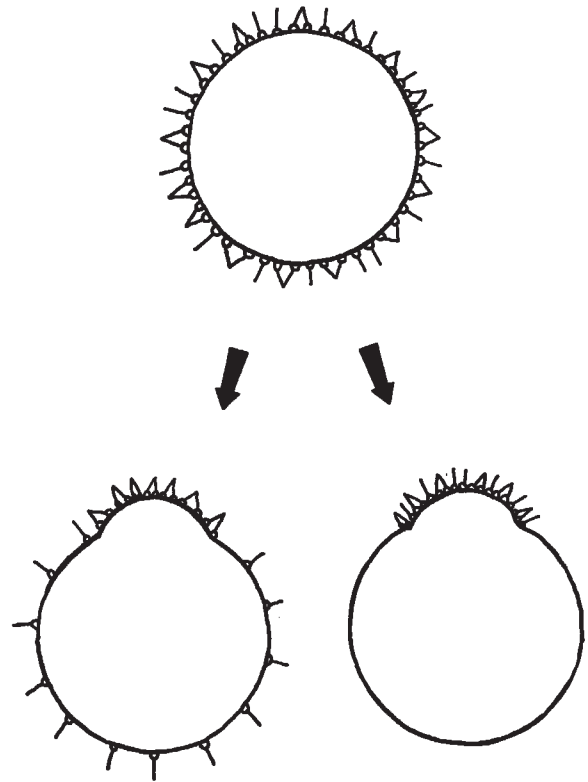
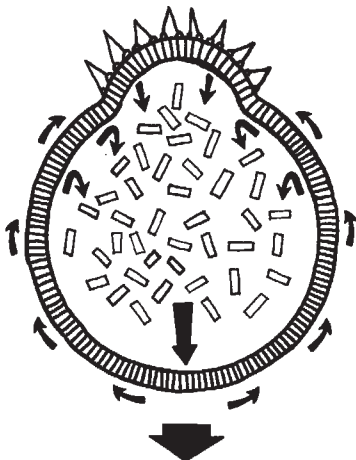


Fig. 2 Diagram of the experiment suggested to distinguish between the membrane recycling theory proposed in this paper and Bretscher's previously proposed theory of directed lipid flow. Lymphocytes are simultaneously exposed to multivalent antibodies against one membrane antigen and to univalent antibodies (Fab fragments) against another of their membrane antigens. The membrane recycling theory predicts that both multivalent and univalent antibodies should accumulate together in the same cap, while Bretscher's hypothesis leads to the expectation that the multivalent antibody should form a cap, while the univalent antibody should remain dispersed.

This directional flow of plasma membrane is envisaged as being responsible for locomotion as well as for capping. Any materials (antibodies, lectins, or small solid particles) which become attached to the surface of a tissue cell undergoing such membrane flow should be carried along to the uropod area of membrane disassembly. Being unable to dissolve in the cytoplasm, these attached materials should remain at the surface, accumulating there to form the familiar "cap". If the attached antibodies and lectins are bound strongly enough to certain components of the membrane, then these components should also be trapped at the surface and accumulated at the cap. Unbound membrane components would, of course, continue to be recycled without accumulating in the cap, though they would pass repeatedly through the disassembly area. In this way my hypothesis can account for the selective accumulation of bound antigens without the need to postulate a molecular filter and also explains why the caps are ordinarily located at the rear uropod area of the cell surface.

The postulate that all components of the plasma membrane, including the lipids, can dissolve into the cytoplasm may at first seem highly implausible or even unacceptable to some readers. Considerable biochemical evidence is, however, accumulating that phospholipids are rapidly interchanged between the several membrane systems of cells. For example, there is rapid equilibration of isotopically labelled phospholipids between mitochondrial and microsomal membranes^{9,10}. This interchange is rapid, reversible and temperature dependent, but does not require metabolic energy, indicating that it represents a true thermodynamic equilibrium. If phospholipids can disassemble from one class of cytoplasmic membrane and reassemble into

another, then it is less surprising that lipid and other membrane components can be disassembled from one part of the plasma membrane and reassembled back into another part of this same membrane, as required by the hypothesis proposed here.

One apparent contradiction to this hypothesis is the observation that univalent antibodies (Fab fragments) and univalent lectins can be bound to lymphocytes without accumulating into caps². However, this observation does not actually contradict the hypothesis because the lymphocytes in question were non-motile and not even attached to a substratum and they were not shown to be simultaneously accumulating other attached materials into caps. If a lymphocyte or other tissue cell could be shown to undergo locomotion without simultaneously accumulating attached materials (even univalent antibodies) into caps, or if cells were shown to cap a multivalent antibody without simultaneously capping a univalent antibody (or lectin) then this hypothesis would be disproved.

How then can one explain the observation that multivalent antibodies do cause capping even on unattached lymphocytes, while univalent ones do not? As a possible explanation, I would suggest that attachment to some sort of surface may be needed for the cell to develop directionality, and to form discrete areas of membrane disassembly and reassembly. In the absence of a solid substratum, the cross linking of the cell surface by multivalent antibodies or lectins may serve as a sort of substitute substratum, stimulating the development of a directional membrane flow and thus capping. This possibility is indirectly supported by the observation of de Petris and Raff¹¹ that cross linking the lymphocyte surface with multivalent antibodies increases the proportion of cells with discrete uroid tail regions, indicative of cell polarity.

These considerations lead to a pair of experimentally testable predictions regarding the accumulation of univalent antibodies and lectins into caps. The first of these predictions is that even univalent materials should cap if the cells are allowed to attach to a solid substratum and to undergo locomotion. Such caps should form at the trailing margins of the cell. Capping of univalent antibodies on motile polymorphonuclear leukocytes has actually been reported¹² but this experiment does not seem to have been undertaken on lymphocytes. The second prediction is that suspended lymphocytes should cap even univalent antibodies, if their surfaces are simultaneously cross linked by multivalent antibodies or lectins. This should occur even if the multivalent and univalent antibodies are specific for completely different and independent surface molecules. In fact, this prediction can be used to test Bretscher's hypothesis relative to the one that I have proposed above.

According to Bretscher's hypothesis, if multivalent antibodies to one membrane molecule and univalent antibodies to another membrane molecule were simultaneously bound to the surface of the same cell, then the multivalent antibodies should accumulate into a cap, while the univalent antibody should remain dispersed over the cell surface. According to my hypothesis, however, both multivalent and univalent antibodies should accumulate together in the same caps. I would suggest that someone should carry out these experiments.

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Lack of effect of naloxone on pain perception in humans

RECENT studies have revealed the existence of endogenous morphine-like compounds in the central nervous system^{1–3} and in the pituitary⁴. These compounds, named *enkephalin*¹ and *C-fragment*⁴, have been reported to produce analgesia when injected intraventricularly in rats and cats^{5,6} and to be antagonised by naloxone which is a potent antagonist of narcotic analgesics. They also have a high affinity for the opiate receptor, which has been discovered in the central nervous system of vertebrates including man⁷, and they can displace naloxone from these receptors^{2–4}. There is substantial evidence that central grey structures in the midbrain are intimately involved in analgesia. Electrical stimulation⁸ and microinjection of morphine^{9,10} in these regions produce analgesia which is antagonised by naloxone. Stimulation of the central grey region in humans¹¹ has been reported to produce marked analgesia which is antagonised by small doses of naloxone¹². In addition, acupuncture analgesia has been reported to be antagonised by naloxone¹³. These findings have led to the hypothesis that there may be an ongoing release within the brain of a morphine-like compound which is partially responsible for pain thresholds. If this were so, then the antagonism of the effects of the endogenous compound by naloxone should affect pain perception. The experiments reported here demonstrate that the perception of experimentally induced pain in normal human subjects is not altered by administration of the opiate antagonist naloxone.

Experiments were carried out on five healthy adult subjects. Each subject was tested on three different days for the effects of intravenous injection of 0.4 mg to 0.8 mg naloxone or a saline placebo, which were administered in a double-blind counterbalanced fashion. Pain was induced by delivering incrementally increasing electric shocks starting at and increasing by 0.2 mA at 1.2-s intervals using a Tursky-Watson type¹⁴ concentric electrode attached to the midpoint of the volar surface of the forearm. The following four parameters were obtained: threshold for sensation, pain threshold, severe pain and maximal tolerance current levels. Subjects were in a semi-recumbent position in a sound-proof room. Respiration rate, skin conductance, heart rate, pulse volume and pupil size were also measured and will be reported in a subsequent paper. The experimental schedule was as follows: 10 min resting and basal recording, one pain test trial session, three consecutive pain trials, 10 min relaxation and basal recording, injection of the drug or placebo and repetition of whole sequence without the trial session.

Figure 1 shows an example of the results obtained from one subject on three different days. The small increase in levels observed after 0.8 mg naloxone is not a consistent finding. These results and those obtained from the other subjects reveal that naloxone does not have a significant effect on any of the four parameters measured in this experiment. The average for the five subjects of the ratios of post-drug level (D) were: 1.06±0.06 (s.d.) for placebo, 1.02±0.18 for 0.4 mg naloxone and 1.03±0.1 for 0.8 mg naloxone.

The lack of effect on perception of pain in our studies suggests that in our experimental conditions there is no significant ongoing release of a morphine-like compound. The amount of naloxone used in our experiments should have been more than adequate to have antagonised any endogenous morphine-like compound present. The dose of 0.4 mg is that recommended to antagonise respiratory depression produced by morphine poisoning and acts within a few minutes¹⁵. Furthermore, 0.1–0.2 mg naloxone has been reported¹² to significantly antagonise analgesia

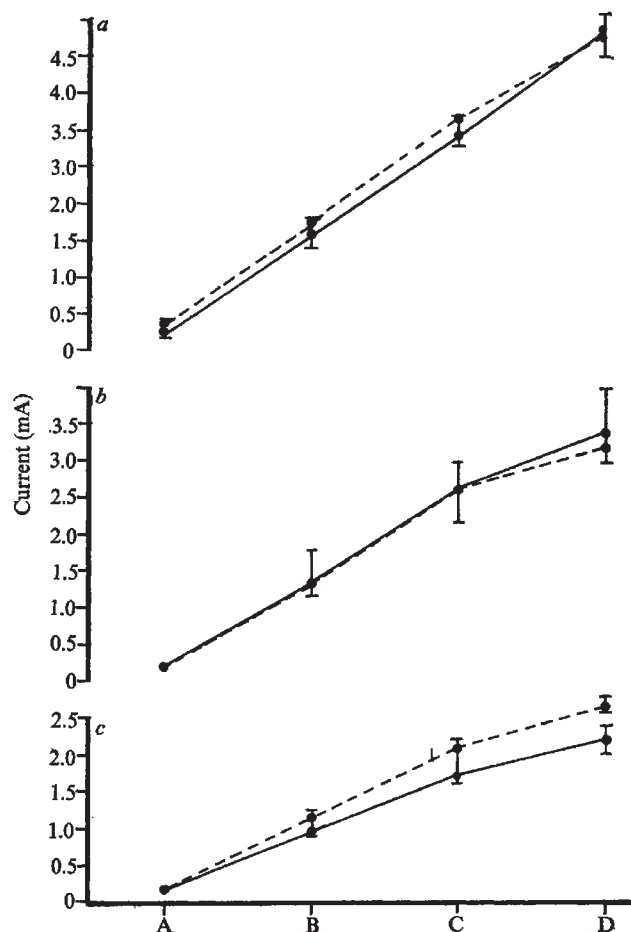


Fig. 1 Results from subject showing lack of effect of placebo (a), 0.4 mg naloxone (b) or 0.8 mg naloxone (c), tested on three different days, on sensation threshold (A), pain threshold (B), severe pain (C), and maximal tolerance (D). Each point is the average of three consecutive trials and the actual range of values for each of the trials is indicated by the vertical 'error' bar; these demonstrate the good repeatability of thresholds from one trial to the next on any one day. Where the ranges of the pre-drug (—) and post-drug (---) results overlap, one combined line has been drawn.

produced by brainstem stimulation in man within 10 min of administration. The method we have used for measuring pain thresholds has been reported reliably to demonstrate the analgesic effects of 10 mg morphine¹⁰. It is true that morphine is used clinically for long lasting pain rather than our brief experimental pain and it is possible that such pains would be more susceptible to modulation by naloxone and an endogenous narcotic. Also in experiments carried out using the same experimental set up (A. El-Sobky, unpublished) used for this study it was found that administration of heroin increased severe pain and maximum tolerance thresholds in heroin addicts. Therefore, it is unlikely that we would have missed any significant effect of naloxone on pain perception. The lack of effect of naloxone on man's pain thresholds which we report here may agree with Goldstein's¹⁷ failure to find a change in rats' avoidance threshold with naloxone.

Since it is unlikely that there is a tonic release of an endogenous morphine-like compound which interacts with pain perception in normal conditions we are left with the problem of explaining the function of opiate receptors and enkephalin in the central nervous system. One possibility

is that they are not involved with pain mechanisms. This would be very surprising in view of the mass of experiments on animals showing numerous connections between opiate receptor concentrations, enkephalin distribution and analgesia sensitivity to morphine administration. Another possibility is that the endogenous morphine-like compound is released only under special circumstances. The existence of special psychological states when pain is not perceived even when the person is severely wounded is well documented in war and some accidents¹⁸.

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Determination of acetylcholine null potential in mouse pancreatic acinar cells

PERHAPS the most important parameter of transmitter action is the value of the membrane potential at which no potential change occurs after excitation. This value is termed the transmitter null (equilibrium) potential (refs 1 and 2 and refs therein). The determination of this value requires the use of two intracellular electrodes, one for recording the cell membrane potential and the other for passing current to set the membrane potential at appropriate levels. In addition it requires the availability of reproducible short lasting applications of transmitter. The recent demonstration that the pancreatic acinus functions as one electrical unit, that is that unimpaired electrical communication between neighbouring acinar cells exists^{3,4}, makes it possible to treat the acinus as one cell. By combining insertion of two electrodes into one acinus with local iontophoretic application of acetylcholine (ACh) from a third extracellular microelectrode, we have measured directly the null potential for the action of a neurotransmitter on mammalian gland cells.

Two separate KCl-filled microelectrodes were inserted very close to each other (20-50 μ m intertip distance, viewed under Leitz stereomicroscope ($\times 160$ magnification)) into surface acinar cells of mouse pancreas *in vitro* as previously described³.

The tip of a third microelectrode filled with 2 M AChCl was placed close ($< 100 \mu\text{m}$) to the impaled acinus. The details of the circuits used for membrane potential measurement, intracellular current injection and iontophoretic ACh application have been described in detail^{5,6}. The composition of the physiological saline solution flowing through the tissue bath was (mM): NaCl, 103; KCl, 4.7; CaCl_2 , 2.56; MgCl_2 , 1.13; NaHCO_3 , 25; NaH_2PO_4 , 1.15; D-glucose, 2.8; Na pyruvate, 4.9; Na glutamate, 4.9; Na fumarate, 2.7. It was gassed with 95% O_2 , 5% CO_2 .

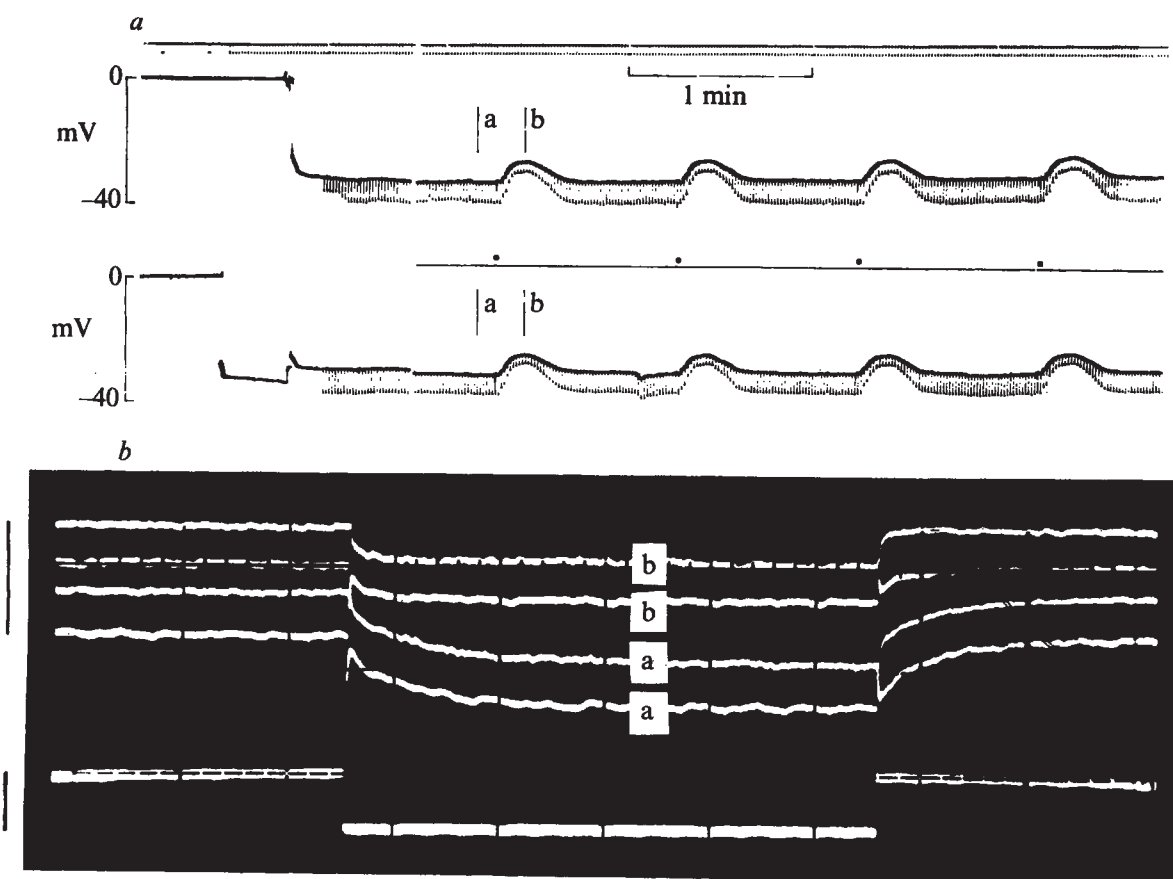
Figure 1 shows the simultaneous recording of membrane potential and resistance from two electrically coupled acinar cells. Iontophoretic ACh applications resulted in reproducible potential and resistance changes of exactly the same size and shape in both cells. If currents (d.c.) of varying size were injected through one of the intracellular microelectrodes, the membrane potential, measured with the other microelectrode, could be set at appropriate levels. Figure 2 shows the marked effect of changes in resting potential on the ACh-evoked potential change. The size of the ACh-evoked depolarisation was clearly reduced as the resting potential was diminished and finally reversed into hyperpolarisation at the very lowest level of membrane potential. In the case represented by Fig. 2, the ACh null potential was -10 mV . In four other tissues, values ranging from -5 to -15 mV were obtained.

In the simplest possible model of transmitter action the membrane potential change (ΔV) after excitation is given by

$$\Delta V(t) = \frac{G_s(t)}{G_s(t) + G_0} (e_s - V_m(t)) \quad \text{where}$$

$G_s(t)$ is conductance of activated synaptic region, G_0 conductance of non-synaptic region, e_s transmitter null potential and $V_m(t)$ membrane potential¹. If one inserts figures for ΔV , G_s/G_0 and V_m taken from experiments like that shown in Fig. 1 (for example, for t corresponding to time of maximal ACh-evoked depolarisation) values for e_s of about $+3$ to -1 mV are obtained. The slight discrepancy between these values and those determined directly (Fig. 2) is a consequence of the slightly nonlinear relationship between ΔV and resting membrane potential at low resting potentials. The directly determined ACh null potential of -5 to -15 mV is rather similar to that in the motor endplate¹ where ACh mainly evokes an increase in Na^+ and K^+ conductance. The ACh-evoked potential and resistance change in pancreatic acinar cells is very sensitive to reductions in extracellular Na concentration⁵ and it has been concluded tentatively that the mechanism of action of ACh in the pancreatic acinar cells is to increase Na^+ and K^+ conductance⁴. The experiments reported here conclusively demonstrate

Fig. 1 Effect of short pulses of iontophoretic ACh application on membrane potential and resistance in two electrically coupled acinar cells. The top part shows the pen recordings, and below is an oscilloscope photograph consisting of two exposures at times marked a and b in the pen recordings. The interruption of the pen recordings represents a 5-min interval. The microelectrode measuring the potential represented in the lower part of the pen recording was inserted first. Subsequently a second microelectrode, through which square wave current pulses could be injected, was inserted. The potential measured by this electrode is shown in the upper part of the pen recording. The short lasting current injections gave rise to short lasting membrane hyperpolarisations, both in the cell into which the current was injected (upper trace) (a circuit was used to compensate for potential changes caused by the current passage through the electrode tip resistance⁶, in the beginning of the record the tip resistance compensation is imperfect and is corrected) and in the neighbouring cell (lower trace). The time course of the polarisations is seen in the oscilloscope photograph (calibration: horizontal 20 ms, vertical 10 mV and $2 \times 10^{-8} \text{ A}$). The coupling ratio (current pulse-induced potential change in the cell of current injection divided by electrotonic potential change in neighbouring cell) is 1. In the event marker trace in the middle are shown signals marking the periods of ejecting current through the extracellular AChCl electrode. A retaining d.c. current of $2 \times 10^{-8} \text{ A}$, to prevent spontaneous leakage of ACh, was used and the ejecting current pulses were of 0.5 s duration and $6 \times 10^{-8} \text{ A}$ strength.



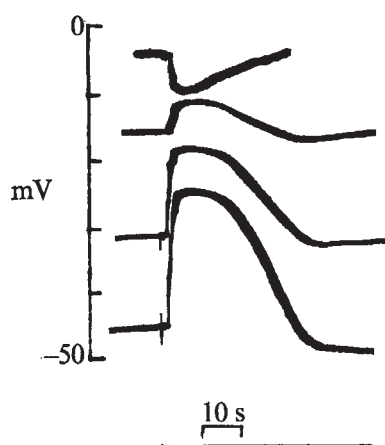


Fig. 2 Effect of short pulses (constant size and duration) of iontophoretic ACh application on membrane potential recorded at four different values of resting membrane potential. The spontaneous resting potential was -31 mV. Higher and lower resting potentials were obtained by passing d.c. hyperpolarising or depolarising currents, respectively, through a microelectrode inserted into a neighbouring cell. A d.c. retaining current of 2×10^{-8} A was applied to the extracellular AChCl electrode and ejecting current pulses of 0.5 s duration and 6×10^{-8} A size were applied at time of marker signal in bottom trace.

that ACh acts on the pancreatic acinar cell membrane by increasing ion conductance and are consistent with the hypothesis that the ion species involved are Na^+ and K^+ .

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Continuous conduction in demyelinated mammalian nerve fibres

MYELINATED nerve fibres conduct in a saltatory fashion, with sites of inward membrane current restricted to the nodes of Ranvier¹. Loss of myelin causes long delays in the internodal conduction time, extreme refractoriness and conduction block². In the large rat ventral root fibres studied the slowed conduction always remained saltatory, with discrete sites of inward current, but the limited spatial resolution of the method used prevented conclusions about whether any membrane beyond that of the original nodes was excited. To find out more about the internodal axon membrane exposed by demyelination, we have used an improved technique of external longitudinal current analysis. A smaller electrode separation ($120 \mu\text{m}$ as against 400 - $600 \mu\text{m}$) has improved the spatial resolution³, and signal averaging has made possible recordings from smaller axons. We have found that in these fibres not only is internodal axon membrane electrically excitable, but that it can support continuous conduction across a demyelinated internode. This result provides a new basis for interpreting the effects of demyelinating disease.

Longitudinal current records from a normal fibre together with their relationships to three nodes are shown in Fig. 1;

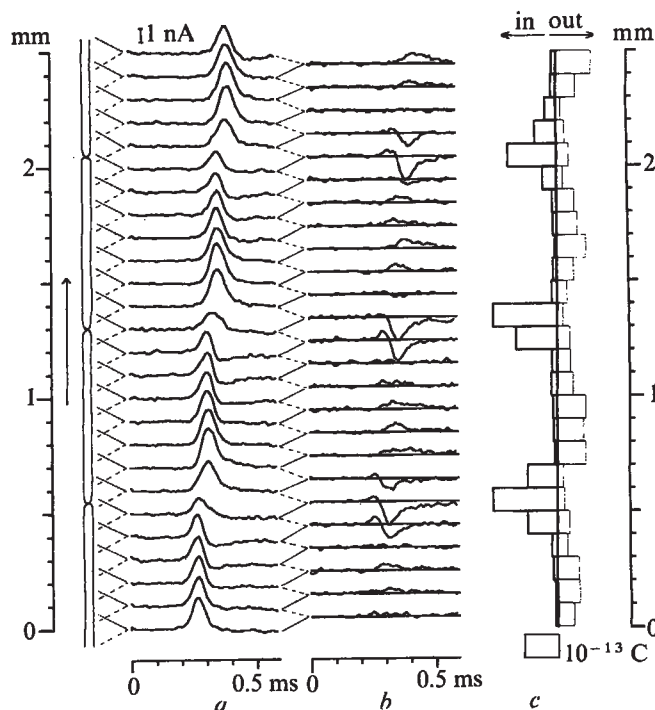
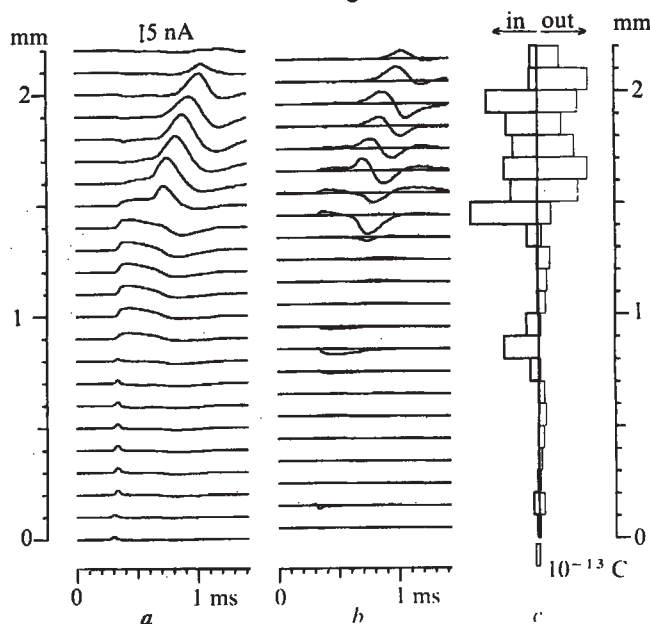


Fig. 1 Action currents in normal ventral root fibre, showing saltatory conduction. *a*, Average longitudinal currents (1,024 sweeps) recorded from two platinum wires, $120 \mu\text{m}$ apart, which were slid $100 \mu\text{m}$ along the root between averages. The approximate positions of the electrodes with respect to the single active fibre are indicated on the left, with the sites of the nodes of Ranvier inferred from the records. The arrow indicates the direction of propagation of the impulse. *b*, Membrane currents (inward current downwards) obtained by subtracting longitudinal currents as indicated. *c*, Inward (thick line) and outward (thin line) current integrals calculated from (*b*).

the membrane currents were calculated by differencing adjacent longitudinal currents as done by Huxley and Stampfli¹. Also shown are the time integrals of inward and outward currents at each position, which correspond to the total charge displaced in either direction for each $100 \mu\text{m}$ of nerve. These records confirm that in normal rat myelinated fibres inward current is confined to the nodes, and

Fig. 2 Action currents in ventral root fibre partially demyelinated with diphtheria toxin, showing stretch of continuous conduction. Explanations of (*a*), (*b*) and (*c*) are as for Fig. 1, but with different scaling.



provides a control for comparison with demyelinated fibres. Figures 2 and 3 are corresponding records for two fibres from animals receiving intrathecal injections of diphtheria toxin 6 d previously, in which the action potentials were propagated in a continuous fashion for about 500 μm . This conduction is termed continuous, because (1), there is a continuous change in latency of the longitudinal currents from one position to the next, in contrast to normal saltatory latency shift, and (2) each 100 μm of nerve in turn becomes a source of inward current, indicating a continuous progression of excitation. The time integrals of inward and outward current reveal that the propagation though continuous, is not even. They also show that at least as much charge crosses the axon membrane in the inward direction along the newly bared internode as at the adjacent nodes.

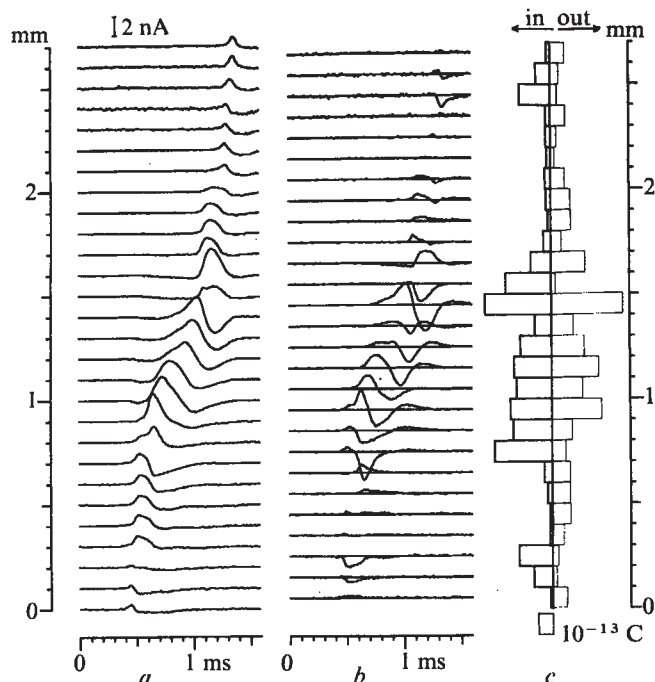


Fig. 3 Action currents in partially demyelinated fibre, showing stretch of continuous conduction and resumption of saltatory conduction. Explanations of (a), (b) and (c) are as for Fig. 1, but with different scaling.

We have recorded five examples of continuous conduction in fibres with internodal spacings in the range 500–850 μm . In three cases (including the two illustrated) the length of continuous conduction was about 500 μm , and the velocity corresponded to roughly 1/20th of the velocity expected for normal stretches of the same fibre. This figure is similar to the ratio of velocity of continuous conduction in the amyelinated roots of dystrophic mice to normal controls^{4,5}. The remaining two cases were of conduction continuous over distances less than 300 μm . Our failure so far to observe such continuous conduction in demyelinated fibres with internodal distances greater than 850 μm (corresponding to an external fibre diameter of about 6 μm), and the previous failure to observe the phenomenon at all², indicates some difference between larger and smaller nerves. It is by no means clear, however, that internodal excitability varies with fibre size, since differences in pathology or geometry could also account for the restriction of continuous conduction to small fibres in our experiments.

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Epstein-Barr virus genome in infectious mononucleosis

THE seroepidemiological evidence¹ linking the Epstein-Barr virus (EBV) causatively with infectious mononucleosis is compelling, but there are many questions concerning the precise nature of the relationship between virus and disease² and the immunological responses that limit the disease^{3,4}. For example, until recently it remained unproven that the transforming agent excreted from the throat during acute and convalescent infectious mononucleosis is EBV specific. Lymphocytes from the umbilical cord can be transformed into continuous cell lines by exposure to the virus excreted from the throat. New evidence presented here and data published recently show that such transformed lymphocytes contain EBV genetic material as well as a virus-specific nuclear protein⁵ even though such cells, unlike those isolated from the peripheral blood, do not shed virus. Another question, persistent and crucial, has been whether the EBV originally derived from Burkitt's lymphoma in Uganda and the EBV associated with infectious mononucleosis are identical. We show here that the viral DNA in human lymphocytic lines derived from infectious mononucleosis partially lacks homology to EBV DNA. Such differences imply that an extensively deleted, defective EBV genome is retained in lymphocyte lines established from infectious mononucleosis or that the cell lines harbour different strains of EBV with up to 35% heterologous DNA sequences.

Students in the infirmary of the University of North Carolina at Chapel Hill were diagnosed as having mononucleosis on the basis of fever, lymphadenopathy, sore throat, splenic enlargement, atypical lymphocytosis and a positive Monospot test (Ortho Diagnostics). The diagnosis was confirmed by the presence of antibodies to EBV capsid antigen (VCA) and to early antigen (EA) as well as by the other data presented in this communication.

Complementary RNA-DNA hybridisation was carried out on duplicate membranes on which had been immobilised 20–80 μg of the cellular DNA to be analysed. Tritiated EBV-specific complementary RNA (cRNA) that was synthesised *in vitro* on a template of EBV DNA with DNA-dependent RNA polymerase from *Escherichia coli* was hybridised to the denatured cellular DNA as described before⁶. The specificity of the ³H-EBV cRNA and the sensitivity of the test have been defined; as few as two EBV genome equivalents per cell can be detected.

DNA-DNA renaturation kinetics analyses were carried out with EBV DNA tritiated *in vitro* as previously described⁷. The reassociated DNA was distinguished from unrenatured DNA by digestion with S1 single-strand-specific exonuclease derived from *Aspergillus oryzae* as described before⁸. We express results as C_0t values (mol s^{-1}) in which C_0 is the concentration of double-stranded DNA; estimates of EBV genome numbers were based on the C_0t_1 values⁸.

In Table 1 are some of the data from a series of patients with proven mononucleosis. Lymphoblastoid cell lines were established from the peripheral blood of the patients after 3–4 weeks of culture *in vitro*. In addition to EBV capsid and early antigens, each of these cell lines contained EBV DNA as shown by cRNA-DNA hybridisation.

Filtered throat washings from the same patients when applied to umbilical cord leukocytes regularly led to the establishment of lymphoblastoid cell lines. Cord lymphocytes fail to proliferate if they are not exposed to the throat washings⁹. These transformed cells do not contain capsid or early antigens (refs 10 and 11 and Table 1). The hybridisation data show that there is EBV genetic material in the cell lines which suggests the

Table 1 Lymphoid cell lines derived from the peripheral blood of patients with infectious mononucleosis or from cord blood leukocytes transformed with throat washings

Patient	EBV anti-body titres		Cell lines from peripheral blood			Cord blood leukocytes transformed with throat washings		
	VCA	EA	No. of genome equivalents per cell*	% Cells with VCA	% Cells with EA	No. of genome equivalents per cell*	% Cells with VCA	% Cells with EA
LH	40	ND	53	1.1	1.7	ND	ND	ND
DT	160	160	20	0.4	1.2	15	0†	0
MB	320	80	37	0.7	1.1	12	0	0
NW	160	160	29	0.7	1.0	11	0	0
KB	320	160	39	0.9	1.3	11	0	0
EB	160	160	34	0.8	1.5	10	0	0

*Determined by RNA-DNA hybridisation with ^3H -EBV-specific cRNA as probe. The specific activity of the cRNA was 6×10^6 c.p.m. μg^{-1} ; an input of 10^5 c.p.m. of cRNA was applied to duplicate samples of 20–50 μg of cellular DNA as described before⁸.

†Less than 1 in 10^5 .

ND, not done. Reciprocals of serum dilutions are given.

virus-specific nature of the transformation. Such cells also contain EBV nuclear antigen (EBNA)¹⁰. The relative number of genome equivalents found in the lines is consistently less than that found in peripheral blood cell lines. Explanted peripheral blood cells sometimes contain large numbers of EBV genomes

and may even replicate virus¹³; this does not occur with the transformed cord cell lines.

Finally, virus shed from one of the peripheral blood cell lines (PB6) could transform cord blood lymphocytes. The secondarily transformed cells also contained a relatively small amount of EBV DNA, but no detectable capsid or early antigens. This last finding indicates that peripheral lymphocytes when transformed into cell lines can shed biologically active EBV.

Perhaps not all the DNA sequences found in the genome of the standard lymphoma-derived P3HR1 virus¹³ exist in the mononucleosis cell lines. Another possibility is that the cord blood lymphocytes, which contain fewer copies of viral DNA and do not express viral structural antigens, do not contain as full a representation of the EBV genome as the cell lines derived from peripheral blood.

Figure 1 shows an analysis of the DNA extracted from pairs of lymphoid cell lines, one from peripheral blood (PB), and one from cord blood cells that had been transformed by throat washings (TW). Each pair of cell lines, PB and TW20 and PB and TW16, derive from materials from a single patient. We found that neither of the peripheral blood cell lines contained all the viral DNA sequences of the prototype EBV genome. The PB20 cells lacked 30–35% of the homologous viral DNA, and the PB16 line lacked 25–30% of the homologous sequences found in the index viral DNA. Nevertheless, both of these cell lines did produce EBV capsid antigens and early antigens. This means either that the whole of the EBV genome is not needed for the coding and translation of these antigens, and that the genome is defective, or that full or unit-length viral DNA is present in the two lines, but that 25–35% of this viral genome contains heterologous DNA sequences.

Another conclusion from these data is that cord blood lymphocytes transformed by the throat washings contain approximately the same viral DNA sequences as are found in the cell lines derived from peripheral blood. Apparently the cord blood lymphocytes do not necessarily select for defective genome. The reason for the sharply restricted expression of the genome contained in the cord cell lines is therefore not apparent.

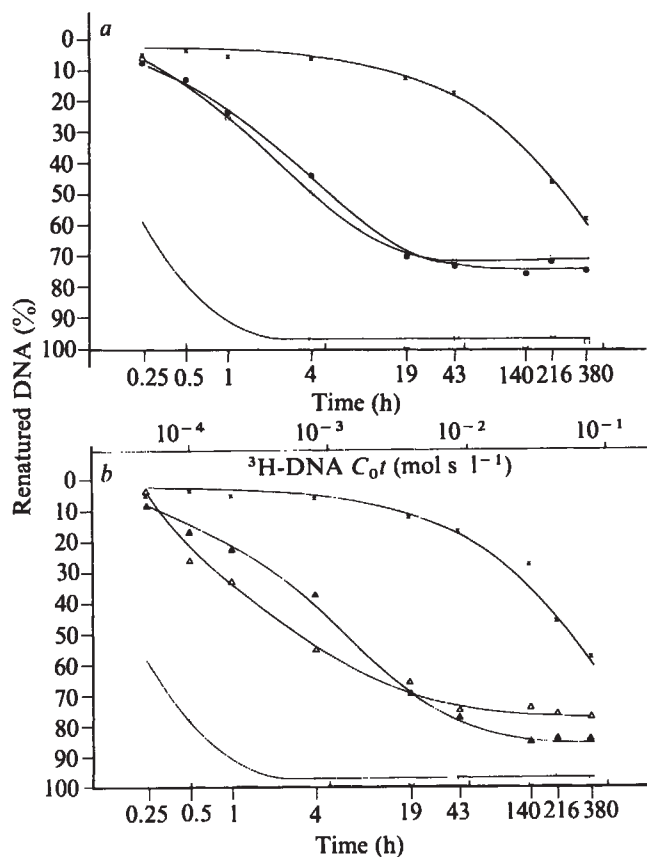
Cord cells transformed by cell-free extracts from a peripheral blood cell line (PB6) contained about 80% of the DNA sequences found in the index viral genome (Fig. 2a).

Figure 2a shows the results of analysis of the DNA of EBV-transformed marmoset cells. This line originated from marmoset lymphocytes that had been transformed by throat washings obtained from a patient with infectious mononucleosis¹⁴. Here approximately 40% of the DNA sequences found in the P3HR1-derived virus are not present in the marmoset line.

An analysis of Raji DNA is also shown (Fig. 2b). The completeness of reannealing of the homologous DNA approaches 100%. This is expected as Raji cells are thought to contain unit-length viral DNA¹⁵ and can be induced with IUdR to make virus¹⁶.

Understanding of the pathogenesis of infectious mono-

Fig. 1 Incomplete homology to the EBV genome of the viral DNA sequences in lymphocyte lines from infectious mononucleosis. Determinations were made by DNA-DNA renaturation kinetics analysis. *a*, $\times$, ^3H -EBV DNA (0.02 μg) + calf thymus DNA (2 mg); $\circ$, PB20 DNA (1.1 mg) was from a cell line derived from peripheral blood; $\bullet$, TW20 DNA (1.8 mg) was a cord lymphocyte line transformed by throat washings from the same patient; $\square$, HR1K DNA (2 mg) was homologous DNA from the EBV-producing cell line that was the source of the index EBV DNA. *b*: Δ , PB16 DNA (1.5 mg) was from a cell line established from the peripheral blood of another patient with infectious mononucleosis; $\blacktriangle$, TW16 DNA (1.5 mg) was from a cell line produced by exposure to throat washings from the same patient. Other symbols as in (*a*). Labelled EBV DNA (0.02 μg , specific activity 1.8×10^6 c.p.m. μg^{-1}) was present in all renaturation mixtures. The total concentration of DNA in all mixtures was brought to 2 mg by addition of calf thymus DNA before denaturation and reannealing.



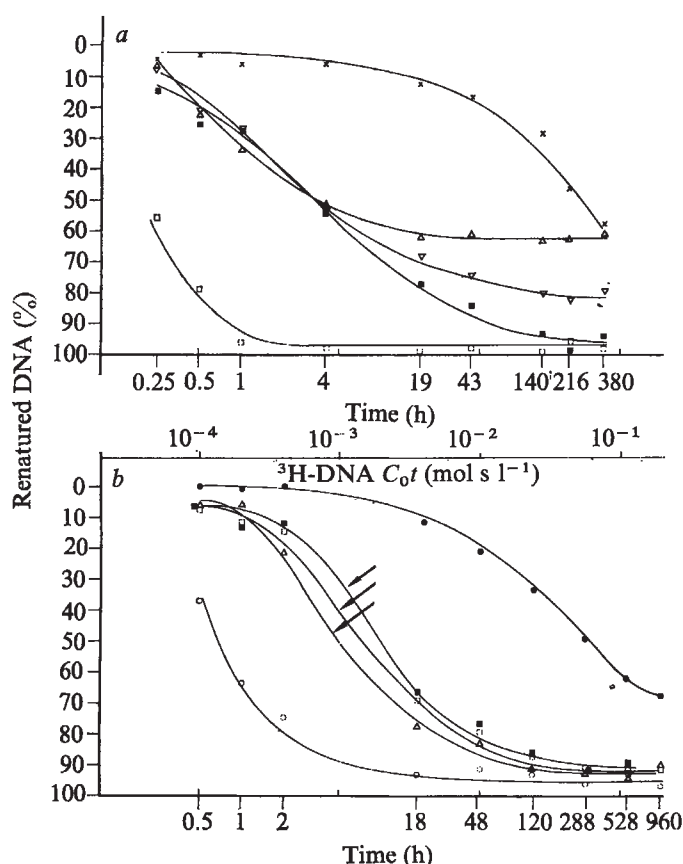


Fig. 2 Extent of homology of the viral DNA sequences found in various lymphoblastoid cell lines. *a*, Δ , B95-8 DNA (2 mg) was from a marmoset cell line transformed by human EBV; ∇ , PB6 DNA (1.3 mg) was from a human cord cell line transformed by virus from a cell line established from the peripheral blood of a patient with infectious mononucleosis; $\times$, ^3H -EBV DNA; $\blacksquare$, Raji DNA; $\square$, HR1K DNA. *b*, Three concentrations of Raji DNA (1, 0.42 mg; 2, 0.56 mg; 3, 0.84 mg), a human lymphoblastoid cell line derived from an African Burkitt's lymphoma; these cells contained fully homologous EBV DNA. $\bullet$, ^3H -EBV DNA; $\circ$, HR1K DNA (2 mg).

nucleosis is still deficient. The virus apparently replicates somewhere in the oropharynx, including perhaps the parotid gland¹⁷. The precise site and cell type in which the virus replicates, however, are not known.

Earlier analyses⁸ suggested that all the P3HR1 EBV DNA sequences were present in three different IM cell lines, but our results obtained with different cell lines show clear differences. Up to 35% of the EBV DNA sequences were missing in cell lines established from peripheral blood as well as in cord blood cells transformed with fresh virus isolates. We do not know whether the molecular weight of the homologous DNA in the cell lines was 35% less than that of virion DNA. Defective genomes might be more liable to persist in lymphocytes, although clearly some lymphocytes contain replicative amounts of genome; explantation of peripheral blood cells can lead to shedding of virus.

In the case of the B95-8 virus, a fuller analysis has been possible. Pritchett *et al.*¹⁸ showed that this virus lacked at least 15% of the DNA sequences found in the P3HR1 EBV, yet the molecular weight of the B95-8 virus DNA was the same as that of EBV. The missing sequences were thought to be made up of repetitions of DNA sequences found in EBV; no heterologous sequences were detected.

Are there strain variants of EBV? The missing sequences in EBV associated with infectious mononucleosis may be replaced by heterologous DNA sequences so that there are different strains of EBV bearing extensive but not complete homology to the EBV derived from an African lymphoma. Epidemiologi-

cally and pathogenetically this is an attractive possibility as brought out recently by Miller *et al.*¹¹, but we cannot distinguish such heterologous viral DNA sequences because of the lack of any other EBV strain in the quantity needed for reciprocal homology studies.

Nasopharyngeal carcinomas also contain viral DNA only partly homologous to EBV¹⁹. Recent analysis of herpes simplex type 1 and type 2 DNA by restriction endonuclease digestion and similar analyses of cytomegalovirus DNA demonstrate the existence of numerous strains of these viruses hitherto undefined²⁰⁻²². The biological significance of this diversity is undetermined. In the case of herpes simplex virus both defective genomes and strain variants exist, and we believe this will prove to be the case with EBV, but we are not yet ready to refer to the Epstein-Barr "viruses".

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Photoreceptor membrane carbohydrate on the intradiscal surface of retinal rod disks

RHODOPSIN is an integral constituent of the photoreceptor membrane where it is believed to function as a light-activated gate for the release of ions or other substances. The shape, localisation and orientation of the rhodopsin molecule in the membrane is therefore of great importance in understanding the primary events of light perception. Uncertainties in the present state of knowledge are reflected in the numerous models which localise rhodopsin variously on the internal, external or both sides of the disk membrane. In the last few years it has become increasingly accepted that rhodopsin is an elongated molecule, partially embedded in or entirely spanning the disk membrane. Even this model leaves many uncertainties

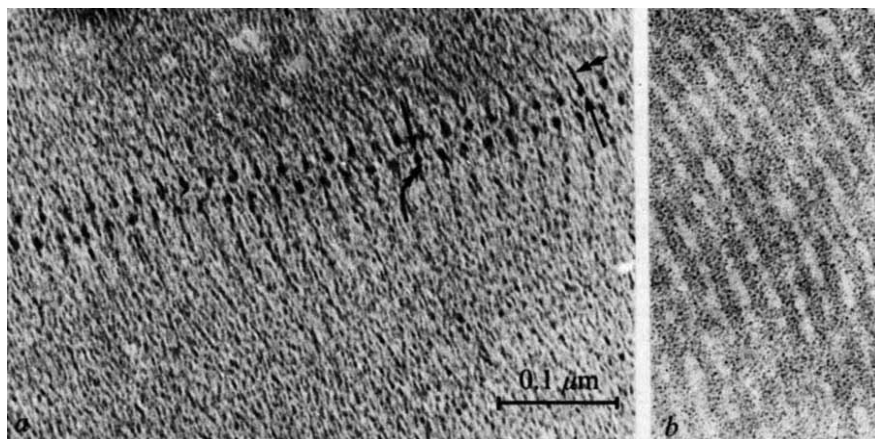


Fig. 1 *a*, Detail of a rod outer segment from a formalin-glutaraldehyde-fixed and Vestopal-embedded frog retina. Carbohydrate reaction (periodic acid-thiosemicarbazide-silver proteinate¹¹) was carried out on thin sections. The dense reaction product is seen inside the membrane loops of the disk margins (dark dots, arrows) whence it can be traced as a thin dense line into the interior of the disks delineating the contact (intradiscal) surface of the membranes (double arrow). *b*, Control section from the same material without oxidation with periodic acid. No reaction can be seen inside the disks.

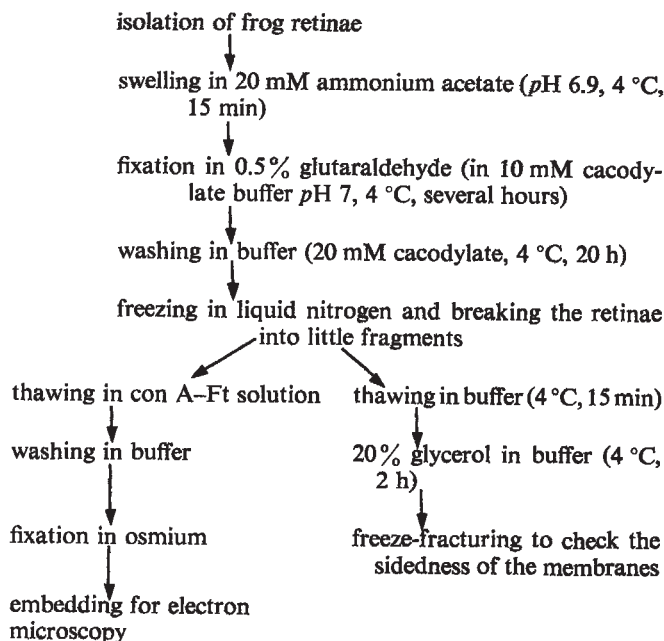
as to the orientation of the molecule in the membrane. Besides the chromophore retinal, the oligosaccharide chain^{1,2} is a good orientation point on the rhodopsin molecule. Since rhodopsin is the dominant protein of the photoreceptor membrane³⁻⁸ where it forms about 85-95% of the total protein content, localisation of the carbohydrate component of the membrane would indicate the location of the rhodopsin carbohydrate moiety. Here I present an electron microscopic histochemical demonstration of the carbohydrate component of the photoreceptor membrane; a part of this investigation was reported briefly earlier⁹.

In the first group, aldehyde-fixed retinæ of frogs (*Rana esculenta*, *Rana pipiens*) were embedded in Epon, Vestopal or glycol methacrylate and electron microscopic histochemical reactions for detection of carbohydrates were carried out on thin sections. Three reactions were based on oxidation of vicinal glycols to form aldehyde groups that were subsequently demonstrated by silver methenamine¹⁰, thiosemicarbazide-

silver proteinate¹¹ or alkaline bismuth¹². Oxidation was omitted in the controls. In addition, the carbohydrate staining by phosphotungstic acid at low pH (refs 13 and 14) was used because of the better resolving power of the method. All electron microscopic histochemical reactions, from which a representative example is shown on Fig. 1*a*, demonstrated a fine reactive line at the contact (internal) surface of disk membranes. The reaction was especially obvious at the disk margin where it was localised at the internal surface of the membrane loop. The reaction was absent in the control specimens (Fig. 1*b*).

In the second part of this work, ferritin-labelled concanavalin A (con A-Ft) was used to detect lectin-binding carbohydrates on the photoreceptor membrane. The con A-Ft was prepared according to de Petris *et al.*¹⁵ and was a gift of Dr D. Lawson (University College, London). A major difficulty in lectin binding was the inability of the voluminous conjugate to penetrate across plasma and disk membranes that was necessary to expose both sides of the photoreceptor membranes to the lectin. The problem was largely solved by a freeze-thawing method as detailed in the following:

Fig. 2 Ferritin-labelled concanavalin A (con A-Ft) is bound to the internal surface of disk-derived vacuoles. No conjugate can be found at the external surface of the vacuoles. This detail represents a portion of an outer segment from a frog retina that was swollen in ammonium acetate, fixed, freeze-fractured and exposed to con A-Ft.



Freeze-fracturing was made in a modified freeze-fracturing device (P. R. Környei and Balogh, to be published) that was based on the cold block principle.

The unilateral localisation of carbohydrates was even more unambiguous with the con A-Ft binding than with the histochemical reactions. The swollen disks appeared as more-or-less

inflated vacuoles. Those disk-derived vacuoles which became patent because of the freezing, fracturing and thawing, contained the con A-Ft bound to the internal (intradiscal) surface of the disk membranes (Figs 2 and 4). No con A-Ft particles were ever found associated with the external (interdiskal, cytoplasmic) surface of the disks, although it was freely accessible to the conjugate because of the broken plasma membrane. The latter showed con A-Ft particles bound to its extracellular surface. No binding was observed using α -methyl-D-glucoside together with the lectin.

It is crucial for evaluating the con A-binding experiment whether the original sidedness of the disk membranes has been retained or not. One way to check this is the known asymmetrical distribution of intramembrane particles on freeze-fractured photoreceptor membranes. As can be seen in Fig. 3, the sidedness of the disk membranes was not altered during the preparative procedure since, similarly to intact disk membranes,

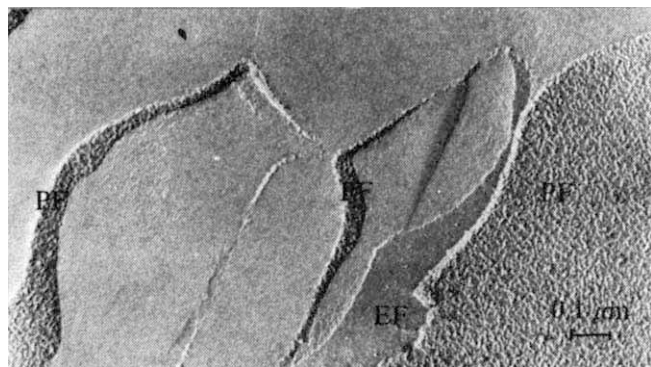


Fig. 3 Freeze-fracture image of swollen disks, treated similarly to those in Fig. 2 (without con A-Ft). The presence of numerous intramembrane particles on fracture face looking towards the lumen of the vacuoles (fracture face of "cytoplasmic" membrane half, PF) indicates that the original sidedness of the disk membranes has not been changed during the preparative procedure. EF, fracture face of the endoplasmic membrane half.

the majority of intramembrane particles was found on fracture face looking towards the interior of the vacuole ("cytoplasmic" membrane half, fracture face PF¹⁶). Another means of recognising the sidedness of the membranes is the known rigidity of the disk edge which preserves its form even after drastic treatments^{17,18}. In our con A-Ft material, disk edges were often observed and their concavity was continuous with the internal space of the disk-derived vacuoles (Fig. 4, arrows). This observation indicates that the disks were only swollen and not everted during the preparative procedure.

Both the carbohydrate reactions on ultrathin sections and the con A-Ft binding on freeze-thawed photoreceptor outer segments showed the presence of a carbohydrate layer at the intradiscal surface of frog retinal rods. The extraordinary molecular simplicity of photoreceptor membranes makes it easy to find the most probable candidate for the carbohydrate layer. Rhodopsin is known to be the dominant protein in outer segment membranes³⁻⁸ (about 9/10 of all membrane proteins being rhodopsin). It is a glycoprotein^{1,2} and binds concanavalin A strongly and specifically^{19,20}. There can be little doubt therefore that of the proteins present in rod outer segment membranes, rhodopsin is primarily responsible for the lectin binding. The contribution of membrane glycolipids to the carbohydrate reaction seems to be negligible, partly because of the low percentage of glycolipids when compared with the rhodopsin content and partly because an intradiscal carbohydrate reaction was present also in our embedded material. But even if we assume a small contribution of glycolipids to concanavalin-binding, it does not make

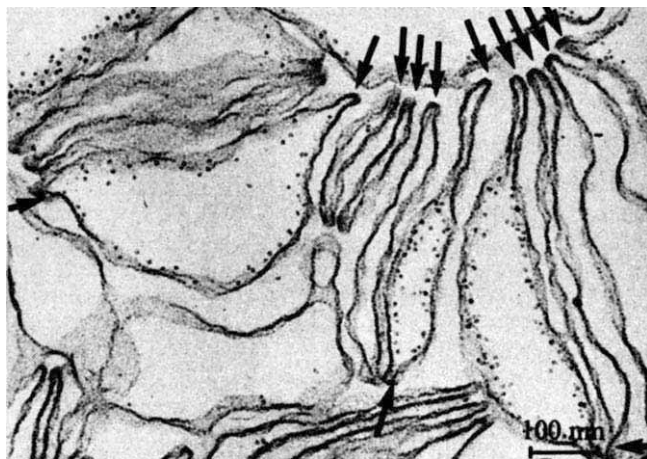


Fig. 4 The same material as Fig. 2 to show persisting disk edges in the con A-Ft experiment (arrows). The membrane loops of the disk edges are directly continuous with the interior of the disk-derived vacuoles indicating the original sidedness of the disk membranes.

localisation of the rhodopsin carbohydrate moiety on the internal disk surface doubtful due to the unilateral localisation of the lectin-binding sites.

Assuming that rhodopsin is responsible for the carbohydrate reaction, the carbohydrate component of rhodopsin must be localised at the intradiscal surface of the disk membrane. This means that the rhodopsin molecule is either situated in the internal half of the membrane or spans the membrane with its extended oligosaccharide chain²¹ protruding into the intradiscal space. Such a localisation of the rhodopsin carbohydrate moiety is supported by observations in which it was shown that the carbohydrate component and the chromophore were not accessible to protease digestion in photoreceptor vesicles, although more than one third of the rhodopsin molecule was digested^{22,23}. At present, it is difficult to compare the present findings with investigations^{20,24} reporting concanavalin binding on the external surface of isolated disks. To correlate the contradicting results it would be necessary to know whether the photoreceptor vesicles in the cited experiments were completely closed and whether all vesicles showed the original sidedness.

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Role of nucleotide hydrolysis in microtubule assembly

MICROTUBULE assembly *in vitro* requires (in normal conditions) that GTP be bound to the exchangeable nucleotide-binding site of tubulin¹⁻³. The bound GTP is hydrolysed during polymerisation and the resulting GDP remains bound to the microtubule while the phosphate is released into the medium. Although hydrolysis normally occurs during polymerisation, microtubules will form in a non-hydrolysable analogue of GTP, guanylyl imidodiphosphate (GMP-PNP)⁴⁻⁶. We have observed⁶, as also has Arai⁵, that microtubules polymerised in GMP-PNP are not depolymerised by concentrations of Ca which completely depolymerise GTP microtubules. We have now found that GMP-PNP microtubules do not display evidence for a rapid equilibrium between polymer and subunit. The irreversible behaviour of GMP-PNP microtubules may explain the insensitivity to Ca and suggests a function for nucleotide hydrolysis during tubulin polymerisation.

The reversibility of polymerisation was studied by dilution of preformed microtubules while constant solvent conditions were maintained. As others have observed⁷⁻⁹, a plot of the extent of polymerisation in GTP against total tubulin concentration intercepts the concentration axis at about 0.2 mg ml⁻¹ (Fig. 1). This concentration has been referred to as the "critical concentration" and is the minimum concentration at which the polymer (microtubules) exists. According to the theory of Osawa and Kasai¹⁰, the critical concentration is the point at which the first turn of the polymer helix is stable and can act as a nucleation structure for further growth. The mechanism of microtubule assembly is not known, but it is likely to be much more complicated than the simple condensation mechanism of Osawa and Kasai's theory. Thus the use of the term "critical concentration" does not imply here any specific model of polymerisation. The observation of a non-zero critical concentration, however, is important because it indicates that below this concentration no microtubules exist. In the dilution experiments this can only mean that the microtubules break down

as the protein concentration is lowered, and therefore that they are in reversible equilibrium with subunits (or small aggregates). The complete breakdown of GTP microtubules after dilution has been confirmed by electron microscopy.

When GMP-PNP microtubules are subjected to the same dilution experiment, the data points lie on a straight line with an intercept on the concentration axis which is indistinguishable from zero (Fig. 1). In eight experiments at temperatures between 20 and 37°C the average intercept was -0.07 mg ml⁻¹ and in no experiment was the intercept greater than zero. The failure of GMP-PNP microtubules to break down at very low concentrations up to 1 h after dilution has been confirmed by electron microscopy. At temperatures below about 18°C GMP-PNP microtubules become reversible and Ca sensitive and the concentration curve has a positive intercept. At these lower temperatures the critical concentration of analogue tubules is always less than that of GTP tubules.

Although microtubules formed in GMP-PNP seem to be irreversible, or nearly so, complete polymerisation of tubulin is not observed in the analogue. The "plateau" level of polymerisation in GMP-PNP was always less than in GTP, although the difference varied widely between preparations. The lower level observed in the analogue is probably a result of inhibition of polymerisation by residual amounts of GDP remaining in the preparation. It is also possible that only a fraction of tubulin subunits are capable of polymerisation in GMP-PNP. Neither of these explanations alters the conclusions reached about the properties of GMP-PNP microtubules.

The polymerisation of tubulin and of muscle actin (and most cytoplasmic actins) requires bound nucleotide (ATP or ADP in the case of actin). In both proteins the bound nucleotide triphosphate is hydrolysed during polymerisation, and in both systems the non-hydrolysable imidodiphosphate analogue promotes polymerisation¹¹. The role of bound nucleotide in actin polymerisation has been studied extensively but no clear consensus exists concerning the function of hydrolysis in polymerisation. It has been suggested that hydrolysis is involved in the depolymerisation reaction of actin¹² but the most direct test of this idea, depolymerisation of AMP-PNP F-actin by dilution, has not been performed successfully¹¹.

In the experiments reported here we have demonstrated that microtubules assembled in GMP-PNP are essentially irreversible and that hydrolysis of bound GTP probably promotes the depolymerisation of microtubules. By analogy, it is reasonable to assume that ATP hydrolysis has a similar function in actin polymerisation. The possible benefit of this to the living cell in regulating polymerisation of fibrous structures is apparent. By using the energy of binding of nucleotide triphosphate, rapid assembly can be obtained. Rapid polymerisation, however, may be incompatible with the need to disassemble rapidly the polymer later (such as during mitosis). Hydrolysis is thus used to produce a more readily depolymerisable state. It has been observed clearly in the case of microtubules and is probably also true of actin fibres that various degrees of stability of the polymer exist in the cell. At least some of this variation could be achieved by maintaining the bound nucleotide as the triphosphate. This could be done by the action of a nucleotide diphosphokinase ("transphosphorylase") such as has been observed as a component of tubulin preparations^{2,6}. Future observations on the state of bound nucleotide in stable compared with labile microtubules and microfilaments may determine whether these speculations are valid.

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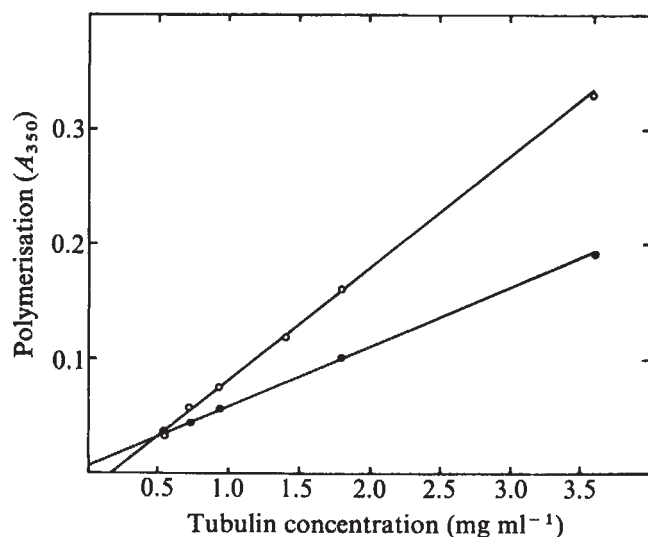
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Fig. 1 Dilution series of GTP and GMP-PNP microtubules. Microtubules were assembled at 35°C in 0.1 M MES buffer (morpholino ethanesulphonic acid) at pH 6.6, 1 mM EGTA, 0.5 mM MgCl₂ and either 1.0 mM GTP or 1.5 mM GMP-PNP (concentrations at which maximum velocity of polymerisation is observed without inhibition). After an apparent plateau level of polymerisation had been achieved, the sample was diluted with the same buffer and the new turbidity level was determined after 5 min. Tubulin was prepared by three cycles of polymerisation and centrifugation in 25% glycerol. After the last centrifugation the pellet was resuspended in buffer and passed through Sephadex G-25 to remove most remaining traces of glycerol and nucleotide. ○, GTP microtubules; ●, GMP-PNP microtubules.



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Protein evolution in cyanobacteria

THERE has been a great deal written in recent years on evolutionary relationships between prokaryotes and eukaryotes^{1,2}, and the possible origin of photosynthesis in eukaryotic cells by endosymbiosis of a cyanobacterium^{3,4}. Molecular methods have been used in an attempt to elucidate the principal events in Precambrian cellular evolution. For example primary structures of plastocyanin⁵, cytochrome *f*⁶, and ferredoxin^{6,7} have been published. These sequences have been compared to primary structures of functionally analogous macromolecules from eukaryotes. Before conclusions on the evolutionary relationships between prokaryotic cyanobacteria and eukaryotic algae and plants can be made from one representative amino acid sequence, it is necessary to evaluate the amount of amino acid sequence variation in proteins isolated from a wide range of cyanobacteria. In this study the amount of variation in plastocyanins and in cytochromes *f* was investigated. The results from complete and partial amino acid sequence determinations on these proteins from a number of cyanobacteria, suggest that the rate of evolution of the proteins in oxygenic photosynthetic prokaryotes is much less than the rates of evolution of corresponding proteins in eukaryotic algae and higher plants.

Plastocyanin and cytochrome *f* are components of the photosynthetic electron transfer chain⁸. Cyanobacteria were chosen which were reasonably diverse but also suitable to culture in the laboratory. The cytochromes *f* were from *Anabaena variabilis*, Kützing; *Plectonema boryanum* 1462/2 (also called *Phormidium luridum* var. *olivaceum*) and *Synechococcus* 6312. The first two are filamentous cyanobacteria and the third is unicellular. The nucleotide base compositions of these strains are 44% (ref. 9), 47% (ref. 9), and 50.2% (ref. 10) G + C respectively. The G + C content of *Spirulina maxima* was 36.6% (M. Herdmann, personal communication) measured on a sample of dried cells from the source in ref. 5. Plastocyanin from *P. boryanum* was also investigated. *A. variabilis* and *P. boryanum* were generally cultured in modified medium C of Kratz and Myers as described previously⁴. *P. boryanum* was also grown in the medium of Allen and Arnon¹¹ and latterly in the medium of Cannon *et al.*¹². Cells of *Synechococcus* 6312 were a gift from R. Y. Stanier.

Isolation of the proteins was, in the initial stages, as described previously^{4,13,14}. After ammonium sulphate fractionation of the cell extract and desalting to 1 mM phosphate buffer, pH 7, the solutions were passed down DEAE-cellulose where only *Synechococcus* 6312 cytochrome *f* was absorbed. Cytochromes *f* and plastocyanins from the other strains were absorbed on to CM-cellulose in the same buffer. The proteins were eluted from the ion-exchange celluloses with buffers of increased ionic strength. The proteins were further purified by absorption on to CM-cellulose at pH 3.9 followed by elution at increased pH and by gel filtration on Sephadex G-75. The protein preparations were checked for purity by polyacrylamide gel electrophoresis¹⁵. Later *Synechococcus* 6312 cytochrome *f* and *A. variabilis* plastocyanin were run in 8% sodium dodecyl sulphate (SDS) gels¹⁷. *A. variabilis* plastocyanin ran partly as a dimer after incubation with SDS in mild conditions although it ran as a monomer during Sephadex gel filtration with phosphate buffers. This phenomenon has been reported for spinach and *Scenedesmus* plastocyanin²⁷.

Cytochrome *f* has been isolated from all cyanobacteria investigated so far in this laboratory but the preparation of plastocyanin has proved much more difficult. No plastocyanin was detected in *Synechococcus* 6312 including one preparation using 500 g fresh weight of cells. Attempts to prepare plas-

tocyanin on a small scale from another two strains of *Synechococcus*, 6307 and *Anacystis nidulans* (*Synechococcus* 6301)¹⁰ and two strains of *Aphanocapsa* (unicellular cyanobacteria) were unsuccessful. A number of attempts to isolate plastocyanin on a large scale from *S. maxima* were also unsuccessful. Initial results of an immunological survey of the culture collection of R.Y. Stanier at Institut Pasteur (my unpublished results) have indicated the presence of plastocyanin in crude extracts of all heterocyst forming cyanobacteria investigated. Extracts from three unicellular species (2 *Aphanocapsa* and 1 *Microcystis*) and a few *Phormidium* and *Pseudanabaena* species show definite cross reaction but tests on a large number of non-heterocyst forming filamentous cyanobacteria (including a species of *Spirulina*) and unicellular species proved negative. There was no immunological cross reaction with an extract of *A. nidulans* although the presence of plastocyanin in this strain has been suggested on the basis of electron paramagnetic resonance signals¹⁸.

The amounts of the proteins isolated were generally very small for sequencing purposes (normal yields were a few mg from 1 kg fresh weight of cells). The sequence information was obtained from proteins isolated from cultures grown over a two-year period. In addition the gifts of some *Synechococcus* 6312 cytochrome *f* from G. Cohen-Bazire and *A. variabilis* cytochrome *f* from D. W. Krogmann were received.

P. boryanum cytochrome *f* was prepared in considerably higher yield (about 20 mg from 650 g fresh weight cells) from a batch grown in the medium of Cannon *et al.*¹², which contains 10-50 times more iron per l than most cyanobacterial media. This success could not be repeated. In an attempt to increase the yield of cytochrome *f*, batches of *A. variabilis* were grown in the presence of diphenylamine (12 mg l⁻¹) in modified Detmer's medium¹⁹. The strain did not grow well in these conditions and much lower yields of cells resulted, although the proportion of cytochrome *f* was increased. The copper content of the cyanobacterial plastocyanins, estimated with bathocuproine²⁰ indicated the presence of 1 mol Cu per mol protein.

Native oxidised plastocyanin and native reduced cytochrome *f* were degraded in a Beckmann, model 890 A automatic sequenator by a 70 min 'Quadrol' double cleavage programme²¹. All three cytochromes *f* tended to wash out of the cup and yields of phenylisothiocyanate derivatives dropped quickly. Identifications proved impossible after 20 cycles. Cytochromes *f* from *Synechococcus* 6312 and *P. boryanum* were later degraded using a single cleavage programme with dimethylbenzylamine²². Identification of phenylisothiocyanate derivatives was carried out as described previously⁴ with the addition that some derivatives were hydrolysed to the free amino acids with HCl²³. The criteria of reliability of sequenator results adopted were generally those of Niall²⁴.

Some of the residues and amide assignments at the amino

Table 1 Percentage similarity matrix for cytochromes *f*

	<i>P. boryanum</i>	<i>Spirulina maxima</i>	<i>Monochrysis lutheri</i>	<i>Porphyra tenera</i>	<i>Euglena gracilis</i>	<i>Alaria esculenta</i>
<i>P. boryanum</i>	—	56	46	54	43	47
<i>Spirulina maxima</i>	56	—	47	53	42	49
<i>Monochrysis lutheri</i>	46	47	—	48	35	45
<i>Porphyra tenera</i>	54	53	48	—	35	67
<i>Euglena gracilis</i>	43	42	35	35	—	39
<i>Alaria esculenta</i>	47	49	45	67	39	—

For the comparison Asx is taken as identical to both Asp and Asn, and Glx to both Glu and Gln.

terminal regions of the cytochromes *f* were confirmed by digestion of small amounts of the cytochromes with trypsin after removal of the haem. The rest of the available material (about 8 mg) was used. Peptides were isolated by established methods for prokaryotic type *c* cytochromes²⁵. Yields of tryptic peptides from many parts of the sequences were very low or zero partly due to the presence of -Asn-Gly- sequences which readily undergo deamidation²⁶.

The alignment of the amino-terminal regions of the cytochromes *f* is shown in Fig. 1. The other parts of the sequences from which peptides were obtained and sequenced are aligned by homology with the complete sequences of *P. boryanum* cytochrome *f* (my unpublished results); *Porphyra tenera* cytochrome *f*²⁶ and the other prokaryotic and eukaryotic cytochromes *f* aligned previously⁵.

The similarity matrix (Table 1) shows that *P. boryanum* and *Spirulina maxima* cytochromes *f* have nearly identical percentages of similarity to each eukaryotic algal sequence taken in turn. The cyanobacterial sequences are 10% closer to each other than the average for all the other sequence comparisons. They are much closer to the red alga (*P. tenera*) cytochrome *f*²⁶ than to the other eukaryotic algal cytochromes. This is interesting in view of the many close similarities between cyanobacteria and red algae²⁷. The partial sequences of the other cytochromes *f* strongly suggest that these numerical relationships are general.

The amino terminal sequence of *P. boryanum* plastocyanin is aligned in Fig. 2 with those of *A. variabilis* plastocyanin⁴, *Chlorella fusca* plastocyanin²⁸ and two examples of higher plant plastocyanins^{29,30}. The partial sequence of plastocyanin

from *P. boryanum* has considerable similarity to the same regions in the other plastocyanins if a deletion at position 13 is considered. Gly-34 (one of the tentative assignments), Val-33 and Met-7 have previously been invariant residues. The similarity matrix (Table 2) gives percentage comparisons between the amino-terminal sequences. This region of *A. variabilis* plastocyanin is slightly closer to the eukaryotic algal plastocyanin than to *P. boryanum* plastocyanin. This finding is valid even if the changes in the previously invariant residues are treated with caution.

Amino acid sequences of cyanobacterial ferredoxin from *Aphanothece* and two strains of *Spirulina*^{6,7} have been compared to primary structures of ferredoxins from eukaryotic algae and plants. Results were obtained similar to those in the present study. The prokaryotic sequences are either not closer or only slightly closer to each other than to the corresponding eukaryotic algal sequences. Where comparisons with higher plant sequences can be made (in the cases of plastocyanin and ferredoxin) cyanobacterial primary structures do seem to be closer to each other than to the higher plant sequences. Ferredoxin sequence comparisons ought to be treated with caution because multiple ferredoxins with quite distinct amino acid compositions have been shown to be present in some plants⁸ and in cyanobacteria^{31,32}.

Fossil evidence indicates that filamentous and unicellular forms of cyanobacteria diverged in the early Precambrian era, about 3×10^9 yr ago³³ and many of these have subsequently evolved little. In the Gunflint Iron formations (1.6×10^9 – 2×10^9 yr old) many representatives of extant cyanobacterial families (Chroococcaceae, Oscillatoriaceae and Nostocaceae)

Fig. 1 Alignment of cytochromes *f*. a, *Synechococcus* 6312; b, *Anabaena variabilis*; c, *Plectonema boryanum*; d, *Spirulina maxima*; e, *Monochrysis lutheri* (chrysophydan alga); f, *Porphyra tenera* (red alga); g, *Euglena gracilis*; h, *Alaria esculenta* (brown alga).

	1	10	20	30
a	Ala-Asp-Ile-Ala-Asp-Gly-Ala-Lys-Val-Phe-Ser-Ala-Asn-Cys-Ala-Ala-Cys-His-Met-Gly-Gly-Gly-Asn(Val)	(Val)Met-Ala-Asn-Lys-Thr-Leu-		
b	Ala-Asp-Ser-Val-Asn-Gly-Ala-Lys-Ile-Phe-Ser-Ala-Asn-Cys-Ala-Ser-Cys-His-Ala-Gly-Gly-Lys			Thr-Leu-
c	Ala-Asp-Ala-Ala-Ala-Gly-Gly-Lys-Val-Phe-Asn-Ala-Asn-Cys-Ala-Ala-Cys-His-Ala-Ser-Gly-Gly-Gly-Gln-	Ile-Asn-Gly-Ala-Lys-Thr-Leu-		
d	Gly-Asp-Val-Ala-Ala-Gly-Ala-Ser-Val-Phe-Ser-Ala-Asn-Cys-Ala-Ala-Cys-His-Met-Gly-Gly-Arg-Asn-Val-	Ile-Val-Ala-Asn-Lys-Thr-Leu-		
e	Gly-Asp-Ile-Ala-Asn-Gly-Glu-Gln-Val-Phe-Thr-Gly-Asn-Cys-Ala-Ala-Cys-His-Ser-Val-Glx-Glx-Glx-Mnl-	Thr-Leu-Glu-Leu-Ser-Ser-Leu-		
f	Ala-Asp-Leu-Asp-Asn-Gly-Glu-Lys-Val-Phe-Ser-Ala-Asn-Cys-Ala-Ala-Cys-His-Ala-Gly-Gly-Asn-Asn-Ala-	Ile-Met-Pro-Asp-Lys-Thr-Leu-		
g	Gly-Gly-Ala-Asp-Val-Phe-Ala-Asp-Asn-Cys-Ser-Thr-Cys-His-Val-Asn-Gly-Gly-Asn-Val-	Ile-Ser-Ala-Gly-Lys-Val-Leu-		
h	Ile-Asp-Ile-Asn-Asn-Gly-Glu-Asn-Ile-Phe-Thr-Ala-Asn-Cys-Ser-Ala-Cys-His-Ala-Gly-Gly-Asn-Asn-Val-	Ile-Met-Pro-Glu-Lys-Thr-Leu-		
	40	50	60	
a				Asn-Ala-Met-
b	-Lys-Lys-Ala-Asp-Leu-Glu-Lys-Tyr			Asn-Ala-Met-
c	-Lys-Lys-Asn-Ala-Leu-Thr-Ala-----Asn-Gly-Lys-Asp-Thr-Val-Glu-Ala-Ile-Val-Ala-----Gln-Val-	Thr-Asn-Gly-Lys-Gly-Ala-Met-		
d	-Ser-Lys-Ser-Asp-Leu-Ala-Lys-Tyr-Leu-Lys-Gly-Phe-Asp-Asp-Asp-Ala-Val-Ala-Ala-Val-Ala-Tyr-Gln-Val-	Thr-Asn-Gly-Lys-Asn-Ala-Met-		
e	-Trp-Lys-----Ala-Lys-Ser-Tyr-Leu-Ala-Asn-Phe-Asn-Gly-Asp-Glu-Ser-Ala-Ile-Val-----Tyr-Gln-Val-	Thr-Asn-Gly-Lys-Asn-Ala-Met-		
f	-Lys-Lys-----Asp-Val-----Leu-Glu-Ala-Asn-Ser-Met-Asn-Thr-Ile-Asp-Ala-Ile-Thr-----Tyr-Gln-Val-	Gln-Asn-Gly-Lys-Asn-Ala-Met-		
g	-Ser-Lys-Thr-Ala-Ile-Glu-Glu-Tyr-Leu-Asp-Gly-Gly-Tyr-----Thr-Lys-Glu-Ala-Ile-Glu-----Tyr-Gln-Val-	Arg-Asn-Gly-Lys-Gly-Pro-Met-		
h	-Lys-Lys-----Asp-Ala-----Leu-Ala-Asp-Asn-Lys-Met-Val-Ser-Val-Asn-Ala-Ile-Thr-----Tyr-Gln-Val-	Thr-Asn-Gly-Lys-Asn-Ala-Met-		
	70	80	90	
a	-Pro-Gly-Phe-Ala-Gly-Arg			
b	-Pro-Ala-Phe-Lys-Gly-Arg-Leu-Lys-Pro-Glu-Glu-Ile-Glx-Asx-Val-Ala-Ala-Tyr-Val-Leu-Gly-Lys-Ala-Asp-	Ala-Asp-Trp-Lys		
c	-Pro-Ala-Phe-Lys-Gly-Arg-Leu-Ser-Asp-Asp-Gln-Ile-Gln-Ser-Val-Ala-Leu-Tyr-Val-Leu-Asp-Lys-Ala-Glu-	Lys-Gly-Trp		
d	-Pro-Gly-Phe-Asn-Gly-Arg-Leu-Ser-Pro-Lys-Gln-Ile-Glu-Asp-Val-Ala-Ala-Tyr-Val-Val-Asp-Gln-Ala-Glu-	Lys-Gly-Trp		
e	-Pro-Ala-Phe-Gly-Gly-Arg-Leu-Glu-Asp-Asp-Glu-Ile-Ala-Asx-Val-Ala-Ser-Tyr-Val-Leu-Ser-Lys-Ala-Gly			
f	-Pro-Ala-Phe-Gly-Gly-Arg-Leu-Val-Asp-Glu-Asp-Ile-Glu-Asp-Ala-Ala-Asn-Tyr-Val-Leu-Ser-Gln-Ser-Glu-	Lys-Gly-Trp		
g	-Pro-Ala-Trp-Glu-Gly-Val-Leu-Ser-Glu-Asp-Glu-Ile-Val-Ala-Val-Thr-Asp-Tyr-Val-Tyr-Thr-Gln-Ala-Gly-	Gly-Ala-Trp-Ala-Asn-Val		
h	-Pro-Ala-Phe-Gly-Ser-Arg-Leu-Ala-Glu-Thr-Asp-Ile-Glu-Asp-Val-Ala-Asn-Phe-Val-Leu-Thr-Gln-Ser-Asp-	Lys-Gly-Trp-Asp		

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
<i>a</i>	Asp	Thr	Val	Lys	Val	Ile	Met	Gly	Gly	(Ser)	Lys	Gly	----	Leu	Val	Phe	Glu	Pro	Ala	Val	Val	Asn	Val	-
<i>b</i>	Glu	Thr	Tyr	Thr	Val	Lys	Leu	Gly	Ser	Asp	Lys	Gly	Leu	Leu	Val	Phe	Glu	Pro	Ala	Lys	Leu	Thr	Ile	-
<i>c</i>	Asp	Val	Thr	Val	Lys	Leu	Gly	Ala	Asp	Ser	Gly	Ala	Leu	Val	Phe	Glu	Pro	Ser	Ser	Val	Thr	Ile	-	-
<i>d</i>	Leu	Asp	Val	Leu	Leu	Gly	Gly	Asp	Asp	Gly	Ser	Leu	Ala	Phe	Ile	Pro	Gly	Asn	Phe	Ser	Val	-	-	-
<i>e</i>	Leu	Glu	Val	Leu	Leu	Gly	Ser	Gly	Asp	Gly	Ser	Leu	Val	Phe	Val	Pro	Ser	Glu	Phe	Ser	Val	-	-	-
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44			
<i>a</i>	-	Lys	Ala	Gly	Asp	Thr	Ile	Gln	Phe	Glu	Val	(Gly)	Gln	(Leu)	Pro	Pro	His	-	-	-	-	-	-	-
<i>b</i>	-	Lys	Pro	Gly	Asp	Thr	Val	Glu	Phe	Leu	Asn	Asn	Lys	Val	Pro	Pro	His	Asn	Val	Val	Phe	Asp	-	-
<i>c</i>	-	Lys	Ala	Gly	Glu	Thr	Val	Thr	Trp	Val	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Ile	Val	Phe	Asp	-	-
<i>d</i>	-	Ser	Ala	Gly	Glu	Lys	Ile	Thr	Phe	Lys	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Val	Val	Phe	Asp	-	-
<i>e</i>	-	Pro	Ser	Gly	Glu	Lys	Ile	Val	Phe	Lys	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Val	Val	Phe	Asp	-	-

Fig. 2 N-terminal amino acid sequences of plastocyanins. *a*, *Plectonema boryanum* 1462/2; *b*, *Anabaena variabilis* (Kützinger); *c*, *Chlorella fusca* (vacuolata); *d*, Potato (*Solanum tuberosum*); *e*, French bean (*Phaseolus vulgaris*). Residues shown in brackets are tentative.

have been identified³³. In more recent rocks (the Bitter Spring formations, 0.9×10^9 yr old) many species of cyanobacteria have been identified at the specific or generic level with extant organisms, including *Spirulina*. If the strains of cyanobacteria for which protein sequence information is available are as evolutionarily diverse as the fossil record suggests, then it is perhaps surprising that their amino acid sequences are not more different. Cyanobacteria were a diverse group of organisms 3×10^9 yr ago³³ and eukaryotic algae diverged in a time measured in hundreds of millions of years³³. There seems to be, however, the same degree of similarity in analogously functioning proteins. It has been postulated that the rate of evolution of a particular protein in a wide range of organisms is approximately constant³⁴. This has been shown for cytochrome *c*, haemoglobin and fibrinopeptide and has enabled phylogenetic trees to be constructed which correspond well with palaeontological evidence³⁴. If cyanobacteria have evolved very little³³ in morphology and physiology since the oxygenic mode of photosynthesis was established in the Precambrian era there is probably little selective value for changes in primary structure of their photosynthetic proteins. Chloroplasts have evolved and diverged in a wide variety of eukaryotes over a much shorter time span than that in which cyanobacteria have

continued to populate similar ecological habitats. The rate of evolution of cytochrome *f* (a type *c* cytochrome) could be expected to be comparable to that of mitochondrial cytochrome *c* which is three "accepted point mutations" per 10^8 yr (ref. 34). The rate of evolution of higher plant plastocyanin is about twice this value³⁵. At these rates of change of amino acid sequence one would expect very little remaining similarity in primary structure of these proteins from diverse blue-green algae (except for a few highly invariant amino acid residues required for a specific function such as binding of the prosthetic group). When the cyanobacterial proteins are compared with analogously functioning proteins in other cyanobacteria or in eukaryotes it would seem that their rates of evolution are much less than the rates of evolution of the proteins in eukaryotic algae and higher plants. The results seem very much against the hypothesis of neutral mutation in protein³⁶. This is the hypothesis that most evolutionary change in DNA is not due to Darwinian natural selection but to selectively neutral mutations which become passively fixed through the action of random genetic drift. These selectively neutral mutations in DNA result in substitutions of amino acids in proteins which do not cause appreciable changes in protein function. In fact this does not seem to be the case here. It would seem that the role of neutral mutation is small and the amount of change in these cyanobacterial amino acid sequences which has occurred seems to reflect the functional differences between the proteins in the variety of organisms. For example it was seen that the cyanobacterial and red algal cytochrome *f* were particularly close.

It is also possible that the pattern of divergence in cyanobacterial primary structures has been obscured by transfer of genetic information coding for these photosynthetic proteins. There is evidence to suggest that this has occurred between distantly related bacteria³⁷, but whatever the reason for the sequence comparisons obtained, protein sequence studies have produced a considerable amount of evidence for the hypothesis of the common origin of oxygenic photosynthesis in prokaryotes and eukaryotes. It seems unlikely, however, that molecular methods will enable construction of phylogenetic trees which would indicate the precise manner in which cyanobacteria evolved into chloroplasts or chloroplast-containing eukaryotes.

I thank Dr R. P. Ambler for invaluable advice and assistance

Table 2 Similarity matrix for plastocyanins

	<i>P. boryanum</i>	<i>A. variabilis</i>	<i>Chlorella fusca</i>	Potato	French bean
<i>P. boryanum</i>	—	49	41	36	33
<i>A. variabilis</i>	49	—	56	36	38
<i>Chlorella fusca</i>	41	56	—	49	46
Potato	36	36	49	—	69
French bean	33	38	46	69	—

The values are expressed as a percentage identity for residues 1–39. The gap in the *Plectonema* sequence has been assigned as a 21st amino acid.

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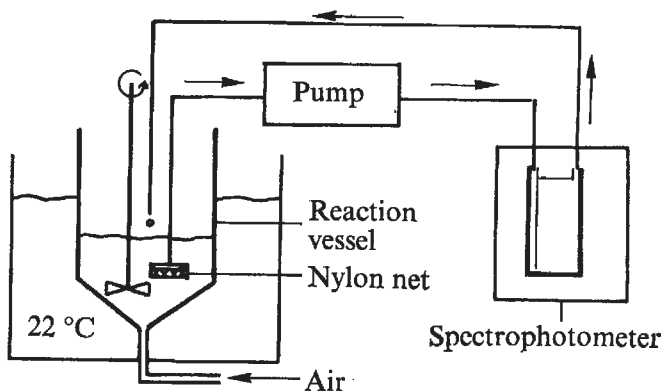
tion. Immobilised microorganisms offer the additional advantage that tedious and costly enzyme isolation is obviated, that the enzyme is usually more stable due to its localisation in its “natural environment”, and that there is usually no need for cofactor. Thus, provided competing reactions can be eliminated, immobilised microorganisms are very promising catalysts.

In this communication we consider especially a property unique to immobilised whole-cell catalysts—the possibility of *in situ* activation of the immobilised enzyme activity. The system studied was the important corticosteroid transformation cortisol $\xrightarrow{\Delta^1\text{-dehydrogenase}}$ prednisolone. The reaction was catalysed by polyacrylamide-entrapped *Corynebacterium simplex*.

C. simplex cells were grown in a medium of 0.25% yeast extract; the Δ^1 -dehydrogenase activity being induced by addition of cortisol to the culture 12 h before collection by continuous centrifugation at 10,000g. The cells (5 g wet weight) were suspended in 20 ml of ice-cold 0.1 M Tris-HCl buffer, pH 7.5, and mixed with 25 ml of ice-cold aqueous monomer solution containing 7.13 g of acrylamide and 0.38 g of *N,N'*-methylene-bis-acrylamide. The mixture was poured into a sandwich-like polymerisation vessel (made of two glass plates 20×20×0.2 cm, spaced 2 mm apart with a piece of latex tubing) and the catalyst potassium persulphate (50 mg) and tetramethylenediamine (100 mg) were added in water (1 ml). Nitrogen gas was bubbled through the suspension and polymerisation started within 2 min. The polyacrylamide gel sheet was fragmented in a blender and the gel granules (average size 0.2 mm) were washed extensively with Tris buffer and then stored at –20 °C at which temperature the preparation was stable for several months.

The 3-ketosteroid- Δ^1 -dehydrogenase activity of the immobilised *C. simplex* was assayed conveniently spectrophotometrically (Fig. 1), and sole product, prednisolone, was identified by thin-layer chromatography. Approximately 40% of the dehydrogenase activity was retained during the immobilisation procedure (all bacteria added were immobilised and no release of bacteria was observed during incubations). Initial experiments revealed, however, that the activity declined rather rapidly on repeated batchwise conversions of high loads of cortisol and this could only be compensated for to a limited degree by addition of the artificial electron acceptor menadione⁶. Instead the stabilising influence of various nutrients and salts was investigated; the results are given in Table 1. In media

Fig. 1 Assay of Δ^1 -dehydrogenase activity. Δ^1 -Dehydrogenase activity of immobilised *C. simplex* was determined in air-saturated 1 mM cortisol. The progress of the reaction was monitored at 285 nm by pumping the supernatant through a flow cell ($\Delta\epsilon = 2,820 \text{ M}^{-1} \text{ cm}^{-1}$). The initial transformation rates listed in Table 1 were determined in an assay composition of 0.5 g of gel granules (wet weight)+9.0 ml of 0.05 M Tris-HCl, pH 7.0, plus, 0.5 ml of 20 mM cortisol (methanol).



New approach to steroid conversion using activated immobilised microorganisms

THE marked increase in demand for contraceptives and anti-inflammatory agents such as cortisol and prednisolone, combined with a diminishing supply of steroid raw materials may lead to shortages of steroid drugs¹. Thus it is important to develop new sources for steroids as well as to devise more efficient means for steroid conversions. Here we report a new approach to steroid transformation in which activated immobilised microorganisms are utilised and which represents a promising alternative to conventional microbial transformation processes.

Immobilised microorganisms have attracted increasing interest as catalysts in the past few years^{2–4}. They have the same operational advantages as those inherent in immobilised enzymes⁵; they are reusable, they are well suited for continuous operation in controlled conditions and further, there is no stringent demand for asepsis during opera-

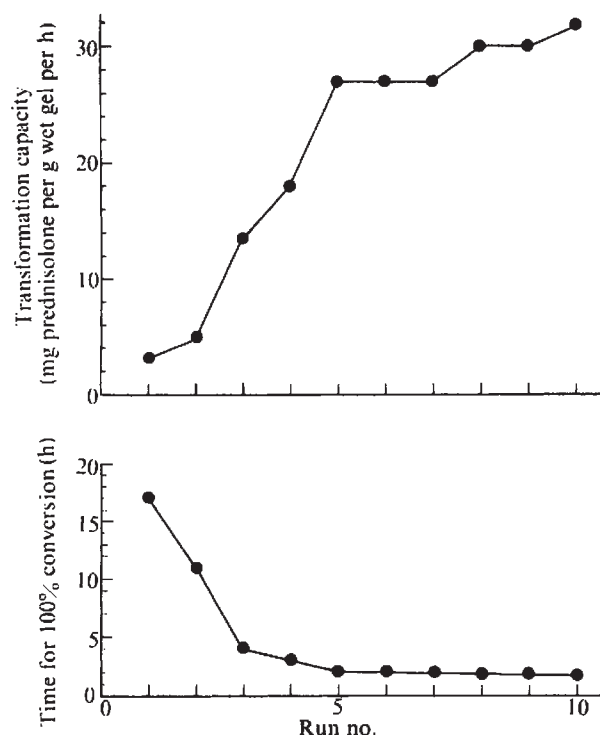


Fig. 2 Repeated batchwise transformation of cortisol to prednisolone. *C. simplex* gel (2.0 g, wet weight) was suspended in 285 ml of 0.5% peptone, pH 7.0, +15 ml of 20 mM cortisol (methanol). The transformation was carried out in the apparatus depicted in Fig. 1. When 100% conversion to prednisolone was reached (indicated by the location of the points), the gel was washed and again incubated with fresh cortisol-containing medium. The whole experiment lasted 4 d.

consisting of water or buffer the activity decreased, whereas in peptone-containing and glucose-containing media the activity was not only preserved but also increased to several times that of the original activity.

The 0.5% peptone medium and the 0.1% peptone + 0.2% glucose medium were selected for further study and an experiment with repeated batchwise transformation was conducted. Both media were approximately equally efficient, and in Fig. 2 the results obtained with the 0.5% peptone medium are depicted. As can be seen, the transformation capacity increased remarkably with each run, thus while in the first batch 100% transformation was obtained after 18 h, the last batch was completed in less than 2 h. The transformation capacity at the end of the experiment was approximately 0.5 g of steroid per d per g of gel (wet weight).

This transformation capacity means that a continuously operating reactor loaded with only 1–2 kg of immobilised *C. simplex* gel would suffice to supply the Swedish demand for 1-dehydrogenated steroids (8 million inhabitants consuming approximately 250 kg of 1-dehydrogenated steroids per year at a value of £5 million). The calculation is based on the assumptions that the catalyst is completely stable, that no decrease in efficiency occurs on scaling up and that the dehydrogenation is the final step in the production of Δ^1 -dehydrogenated steroids. Even if these assumptions are too optimistic, the calculation still serves to give some idea of the very modest reactor dimensions needed even for large-scale production of Δ^1 -dehydrogenated steroids.

Preliminary experiments show that the so-called pseudo-crystallofermentation technique⁷ is applicable also to entrapped *C. simplex*. Cortisol was thus added in an amount (3.6 g l⁻¹) far exceeding its solubility in the medium and was

Table 1 Activating effect of nutrients on the Δ^1 -dehydrogenase activity

Medium	Initial transformation rate (%) after				
	0	2	6	10	16 d
Peptone, 0.5%	100	460	500	650	530
Peptone, 0.1% + Glucose, 0.2%	100	270	230	250	250
Glucose, 0.2%	100	210	170	110	90
K ₂ HPO ₄ , 0.1 M, pH = 7.0	100	70	50	60	60
Tris-HCl, 0.05 M, pH = 7.0	100	100	70	70	30
H ₂ O	100	60	40	40	20

C. simplex gel (0.5 g) was incubated in 9.0 ml of medium as indicated and 0.5 ml of 20 mM cortisol (methanol) was added. The suspension was shaken on a rotary shaker at 25 °C and at 48-h intervals the medium was replaced by fresh cortisol-containing medium. At the times indicated the gel was filtered off, washed and assayed for Δ^1 -dehydrogenase activity as described in Fig. 1.

completely converted at approximately the same rate as in experiments with dissolved cortisol. The product prednisolone, which precipitated out, could be isolated simply by filtration after the rather dense gel granules had been allowed to settle. This technique allows reduction of media volumes by orders of magnitude and thus also of nutrients.

The reason for the observed activation is not known; it could be microbial growth or protein synthesis. Microscopic studies of gel granules revealed that there is at least some increase in the amount of entrapped bacteria. Continuous induction leading to *de novo* synthesis of Δ^1 -dehydrogenase in existing cells is also a plausible explanation. Cell lysis, which might enhance substrate/product transport, is, on the other hand, considered a rather unlikely cause of activation, since increased activity was observed only in media containing nutrients (Table 1).

In situ (re)activation procedures similar to that given here should obviously also be applicable to other processes catalysed by immobilised microorganisms, for example, biotransformation of other steroids and antibiotics as well as other compounds.

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Polymers for the sustained release of proteins and other macromolecules

SINCE the first demonstration that silicone rubber¹ could be used as an implantable carrier for sustained delivery of low molecular weight compounds in animal tissues, various drug delivery systems have been developed. But except for the reports of Davis^{2,3} and Gimbrone *et al.*⁴, there has been little success in the development of slow release agents for large molecular weight compounds. Furthermore, the polymers used in those studies, polyvinylpyrrolidone and polyacrylamide, are often inflammatory in animal tissues and

Table 1 Host response to corneal polymer gel implants

Polymer	No. of tests	Inflammation*		
		None	Mild	Significant
Polyacrylamide, 20%	10	20%	30%	50%
Polyvinylpyrrolidone, 20%	4	0	0	100%
Polyvinylalcohol (unwashed, 1%, 5%, 10%)	20	15%	70%	15%
Polyvinylalcohol (washed, 10%)	6	67%	33%	0
Hydron-S (with and without water in the polymer casting solution)	15	100%	0	0
Ethylene-vinyl acetate copolymer (unwashed)	20	40%	60%	0
Ethylene-vinyl acetate copolymer (washed)	20	100%	0	0

*The criteria were oedema, white cells and neovascularisation as observed through a stereomicroscope. A response was judged to be mild if any one of the three characteristics was detected, and significant if their presence was enough to cause corneal opacity.

usually permit only brief periods of sustained release. We now present a simple method for incorporating various proteins and other macromolecules into non-inflammatory polymers. Sustained release of biochemically active macromolecules has been demonstrated for periods exceeding 100 d.

Sterile polymer casting solutions were made by dissolving polymers in appropriate solvents: Hydron (a polymer of hydroxyethylmethacrylate) in absolute alcohol at 37 °C; ethylene-vinyl acetate copolymer (40% by weight vinyl acetate) in methylene chloride at 37 °C; and polyvinylalcohol by autoclaving in distilled water. Slow release pellets were made by placing a mixture of casting solution and the macromolecule to be released in a conical mould, 2–4 mm in diameter and 1.5 mm deep. Glass moulds were used for the ethylene-vinyl acetate copolymer because the polymer casting solution reacted with plastics causing adhesion. The moulds were dried under a mild vacuum (600 mmHg) overnight causing the solvent to evaporate leaving the macromolecule trapped within the polymer matrix. To retard further release of macromolecules, 'sandwiches' were sometimes made by coating the matrix with pure polymer in casting solution. Coating was achieved by dipping the polymer pellet in a puddle of pure polymer in casting solution and drying. In an alternative method of coating, pure polymer in casting solution was dried in the bottom of a mould, a middle layer of polymer and macromolecule was added and dried, and finally a top layer of pure polymer in casting solution was added and dried. Before use, the dried slow-release pellets were rehydrated by adding a drop of Ringer solution.

To study tissue response to the polymers, sterile polymer pellets (1.5 × 1.5 × 0.5 mm³) were implanted in the eyes of rabbits, by aseptically creating a small pocket in the cornea⁴. Inflammation was judged by stereomicroscopy. The sensitivity of the cornea to inflammatory stimuli as well as its clarity and avascularity make it an excellent site to observe inflammatory characteristics: oedema, white-cell infiltration, and neovascularisation⁵.

Ethylene-vinyl acetate copolymer, exhaustively washed in alcohol, and Hydron caused no inflammation in the cornea (Table 1). Histological sections made 3–4 weeks after implantation showed no foreign cells in the region surrounding the polymers or in other areas of the cornea. In contrast, polyacrylamide and polyvinylpyrrolidone, prepared according to Davis³, were significantly inflammatory. Polyacrylamide has been used in our laboratory for several years⁴, and inflammatory responses have been reduced by various washing techniques (R. Arensman and M. Gimbrone, unpublished). Unwashed polyvinylalcohol pellets also caused inflammation. Washing the polymer exhaustively with alcohol greatly reduced, but did not eliminate, the inflammatory response (Table 1).

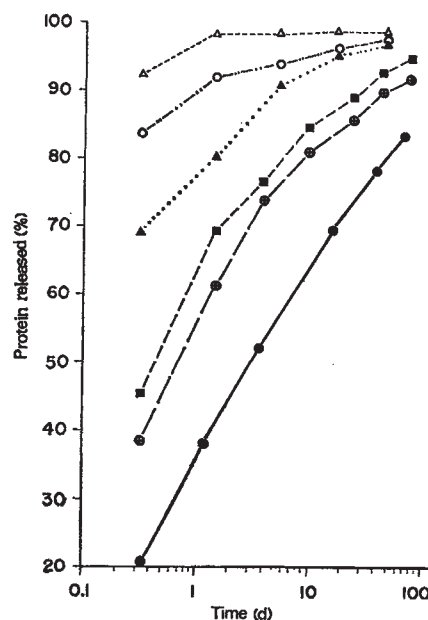
Polyvinylalcohol, Hydron and ethylene-vinyl acetate copolymer were examined for the ability to release soybean trypsin inhibitor (molecular weight 21,000). The polymer-water ratio in the casting solution of polyvinylalcohol was

important in the determination of release rates. The effects of various polyvinylalcohol concentrations in the casting solution on the rate of release of soybean trypsin inhibitor are summarised in Fig. 1. An increase in the polymer concentration in the casting solution significantly decreased the initial rate at which protein was released. A threshold level was apparently reached at about 10% polyvinylalcohol, because further increases in polymer concentration did little to retard release from the pellet. Water was also added to the Hydron-alcohol-protein casting solution. The greater the water-polymer ratio, the greater was the rate of protein release from the pellet.

Figure 1 illustrates two additional features of these slow release systems: (1) a large percentage of protein was released during the first hour of incubation, that is, 'burst effect'; (2) the rate of release was significantly retarded in polymer 'sandwiches'.

Figure 2a shows the release of soybean trypsin inhibitor from the three polymers. Protein was released from Hydron relatively rapidly, more slowly from polyvinylalcohol, and least rapidly from ethylene-vinyl acetate copolymer. The 'burst' was evident for all three systems.

Fig. 1 Release of soybean trypsin inhibitor from polyvinylalcohol. The percentage of polymer in the casting solution was varied. Protein concentration in all casting solutions was 12 mg ml⁻¹. Release characteristics were studied by incubating polymer pellets in small test tubes with lactated Ringer solution at 37 °C. Periodically, pellets were removed to new tubes containing fresh Ringer solution. Protein release from the pellets was assayed by the Lowry method⁶. Polyvinylalcohol: △, 1.2%; ○, 4.8%; ▲, 6%; ■, 10%; circle and cross, 20%; ●, 10% sandwich.



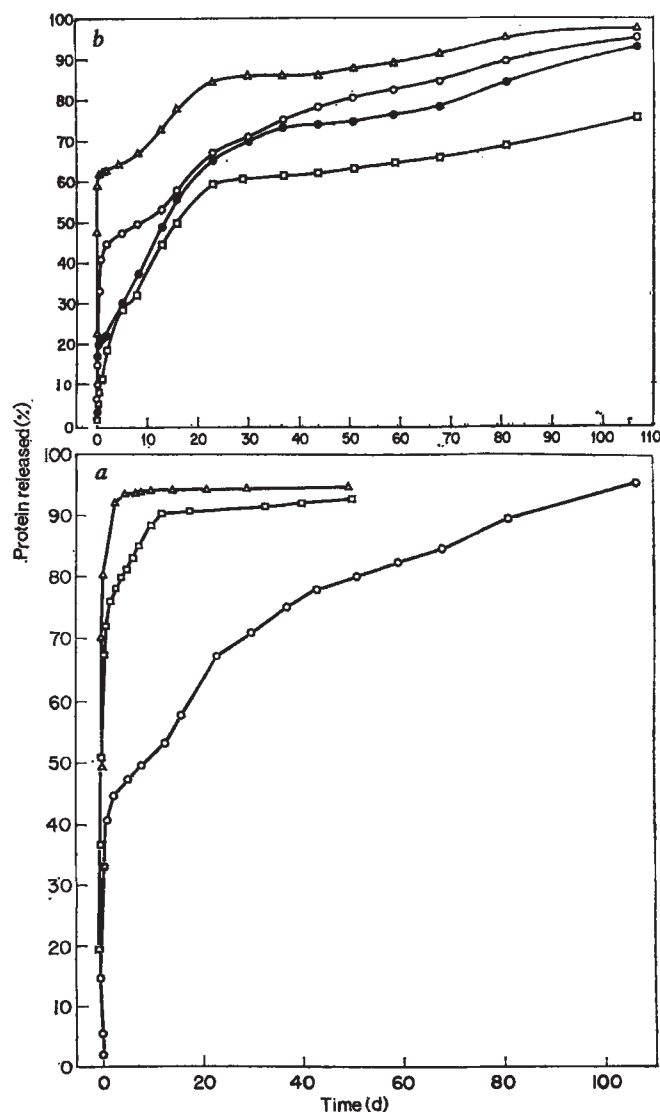


Fig. 2 *a*, Release of soybean trypsin inhibitor from Hydran (Δ), polyvinylalcohol ($\square$) and ethylene-vinyl acetate copolymer 'sandwiches' ($\circ$). Protein concentration in all casting solutions was 50 mg ml⁻¹. Polymer concentrations in casting solutions were 12% Hydran, 10% polyvinylalcohol and 10% ethylene-vinyl acetate copolymer. These concentrations minimised initial rates of release from each of the individual polymer systems and still permitted viscosities low enough so that the solutions were easy to work with. *b*, Release of four proteins from ethylene-vinyl acetate copolymer 'sandwiches'. Protein and polymer concentrations in casting solutions were 50 mg ml⁻¹ and 10%, respectively. Release was studied as described in Fig. 1. Δ , Lysozyme; $\circ$, soybean trypsin inhibitor; $\bullet$, alkaline phosphatase; $\square$, catalase.

Three other proteins, lysozyme (molecular weight 14,400), alkaline phosphatase (molecular weight 88,000), and catalase (molecular weight 250,000) were similarly studied. Their release profiles from ethylene-vinyl acetate copolymer are shown in Fig. 2*b*. Release rates approach zero-order kinetics over periods of 20–100 d. Using standard assays⁷⁻⁹, we also found that this polymer released more than 100 ng per day of heparin, and ³H-thymidine-labelled DNA (isolated from mouse SVT2 cells); and in excess of 100 μ U per day of insulin for periods exceeding 40 d.

To determine whether the polymers were releasing biochemically active material *in vitro*, pellets containing 300 μ g of lysozyme, soybean trypsin inhibitor, and alkaline phosphatase were placed in 20-ml volumes of Ringer at 37 °C. After 2 d of this incubation, each polymer was washed with 100 ml of Ringer for 5 min and placed on an agar diffusion

slide described before¹⁰⁻¹². Formation of zones of activity on these slides indicated the release of at least 100 ng per day of biochemically active material. When this cycle was repeated, new zones continued to form for periods exceeding 100 d with ethylene-vinyl acetate copolymer 'sandwiches', and for 14 and 4 d with polyvinylalcohol and Hydran 'sandwiches', respectively. Furthermore, when the specific activity of alkaline phosphatase was tested before and after incorporation into each of the three polymers, more than 80% of the enzyme escaping from the polymers was found to be active.

To determine whether biologically active macromolecules could be released *in vivo*, ethylene-vinyl acetate copolymer 'sandwiches' containing tumour angiogenesis factor (TAF), a tumour cell extract that induces neovascularisation¹³, were tested. Pellets containing TAF were implanted into 20 rabbit corneas and in every case vessels sprouted from the corneal edge and grew towards the polymer. No oedema or white cells were detected. After 40 d, several of the polymers were removed and vessels disappeared within 21 d. When the same polymers were washed in Ringer solution and transplanted to new corneas, vascular responses were again observed, although rates of vessel growth were somewhat slower than with the initial implants. Also, pellets of polyvinylalcohol or ethylene-vinyl acetate copolymer containing TAF consistently produced neovascular responses when implanted on a 10- or 11-d-old chick chorioallantoic membrane. The polymers alone induced no such response.

These studies show that sustained release of proteins and other macromolecules from polymeric vehicles can be achieved over prolonged periods. The mechanism of release of large molecules from these polymers is not entirely clear. The mechanism, at least for ethylene-vinyl acetate copolymer, does not seem to depend on simple diffusion. The same macromolecules which were released so readily from this polymer after incorporation into its matrix, do not diffuse through a film of the pure polymer. Furthermore, when Dextran blue (molecular weight 2,000,000) was incorporated into ethylene-vinyl acetate copolymer, more than 90% was released in 3 d, in contrast to proteins such as alkaline phosphatase (molecular weight 88,000) where 90% release required nearly 100 d. It can be speculated that because this polymer is permeable to water, proteins deep within the polymer matrix may be driven to the surface by osmotic pressure or capillary action.

There are several possible applications. These polymers may be useful in the development of bioassays for various substances, including growth factors, chemotactic compounds and other informational molecules. The ability of ethylene-vinylacetate copolymer in casting solution to bind to plastics may facilitate new approaches for tissue culture experiments. It remains to be seen whether these polymers will eventually be clinically useful for long term delivery of macromolecules such as insulin, heparin or enzymes.

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Enhancement in the sweetness of sucrose

THERE is considerable interest in the relationship between chemical structure, both configurational and conformational and the sweet response¹ particularly in the case of sucrose (1) since, of all the sweetening agents it is the most widely used. Hitherto, no derivative of sucrose nor any other carbohydrate has shown sweetness substantially greater than that of sucrose, indeed to the contrary sucrose octa-acetate is very bitter, sucrose monoesters are much less sweet and *galacto*-sucrose (2), a C4 epimer of sucrose, is greatly diminished in sweetness². Nevertheless, one of the objectives in our studies on the chemistry of sucrose has been to modify its structure, by chemical means in order to enhance its natural sweetness and in the process prevent metabolism by inhibiting breakdown by invertase. We have now been greatly encouraged by the surprising discovery that a *galacto*-sucrose derivative, namely 1',4,6,6'-tetrachloro-*galacto*-sucrose (3) is intensely sweet, comparable with saccharin but without an unpleasant after-taste.

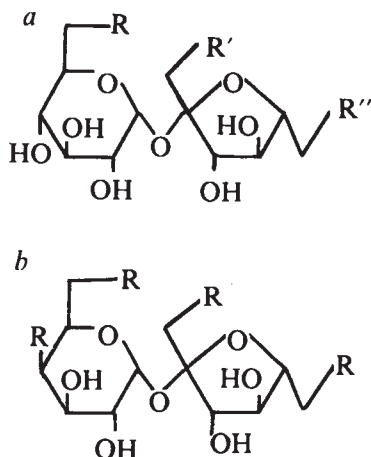


Fig. 1 *a*, Sucrose (1; R = R' = R'' = OH) and its 6,6'-dichloro (4; R = R' = Cl, R'' = OH) and 1',6'-dichloro (5; R' = R'' = Cl, R = OH) derivatives; *b*, *galacto*-sucrose (2; R = OH) and its tetrachloro derivative (3; R = Cl).

Lindley, Birch and Khan² suggested from studies on methyl ethers of sucrose that the equatorial 4-hydroxyl group of sucrose (1) interacts with the taste buds of the tongue by intermolecular hydrogen bonding and that loss of sweetness in *galacto*-sucrose (2) is due to the axial 4-hydroxyl group being masked by an intramolecular hydrogen bond to the ring oxygen. Whilst our tetrachloro derivative (3) is not in disagreement with the latter observation, the 4-chloro substituent could only act as a hydrogen bond acceptor on the taste bud. Likewise they suggested the importance of the 1'-hydroxyl group of sucrose which is supported by our own observations in that 6,6'-dichloro-sucrose³ (4) is less sweet than sucrose, implying that in (3) the 1',4-dichloro substituents are enhancing its sweetness. On the other hand 1',6,6'-trichloro⁴ and 4,6,6'-trichloro⁵ derivatives are more than 10 times sweeter than sucrose but not as intensely sweet as the aforementioned tetrachloro derivative (3). It is highly significant that 1',6'-dichlorosucrose (5) is intensely sweet, like the tetrachloro derivative (3), which

pinpoints the importance of the chloro substituent at the 1'-position on the molecule of sucrose.

If the Shallenberger theory of sugar sweetness⁶ is correct, where two electronegative atoms A and B separated by 2.5-4.0 Å initiate the sweet taste by intermolecular hydrogen bonding between A-H and B and similar groups on the receptor site, then it would seem that the 1'-chloro substituent is the proton acceptor B and the 2-hydroxyl on the glucosyl moiety is A-H, the proton donor. The intense sweetness of the chloro compounds 3 and 5 could also be due in part to their greater lipophilic character than sucrose. The subtle interplay of intramolecular bonding in the sucrose molecule has an important role and the introduction of chloro substituents seems to have reinforced the effect. The sucrose derivatives in which a 6-hydroxyl of the fructofuranosyl unit of sucrose has been replaced by a chloro substituent are resistant to the action of invertase, a β -D-fructofuranosidase (J. Chapman, unpublished results). Hence in the development of alternative sweeteners from sucrose, as for example for application in dietetic food, combining high sweetness with low calorie content, the chloro derivatives are worthy of further evaluation.

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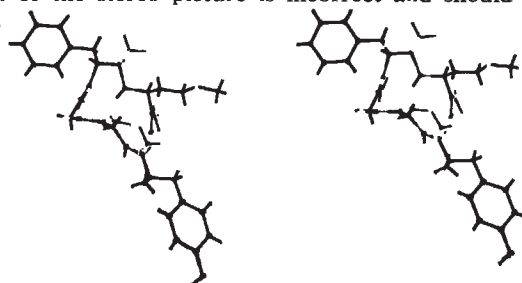
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Erratum

In the article "Conformation of met⁵-enkephalin determined by high field PMR spectroscopy" by B. P. Roques *et al.* (*Nature*, **262**, 778; 1976) the legends to Figs 2 and 3 are transposed. The Figs are in the correct positions. The alignment of the stereo picture is incorrect and should be as below.



Nature Index and Binders

The **Index** for 1975 is now available, price £2.25. Copies of the 1974 index are still on sale, price £3.00. **Binders** for the journal are also available at £8.00 for four (a year of *Nature* fits into four binders).

Postage is included in the above prices. Orders should be sent, accompanied by remittance to Macmillan Journals Ltd, Brunel Road, Basingstoke, Hampshire, England.

reviews

Tackling computer myths

Yorick Wilks

The Thinking Computer: Mind Inside Matter. By Bertram Raphael. Pp. xiii+322. (Freeman: San Francisco and Reading, May 1976.) Cloth £9; paper £4.40.

ARTIFICIAL INTELLIGENCE (AI) is beset by all the difficulties of any immature science. Since so little is agreed among its workers in the way of basic theory and truths, any presentation of it is bound to be inadequate, and that applies especially to any attempt to produce a non-contentious introductory text like the one under review. It is almost inevitable that such a work will be parochial in terms of the research laboratory it comes from, and the areas within the field on which it concentrates. To say this is not to blame the author: in the present state of things in AI it could not be otherwise.

Thus the present book is heavily oriented to the research carried out at the Stanford Research Institute (SRI), its author's home, and is strongest in the areas where SRI is strong: robots, formal approaches to problem solving and vision; and weakest where they are weakest: natural language, high level programming languages, and the sort of speculation about 'knowledge structures' that has been emerging from MIT in the past two years. The book seems to have been written essentially by about 1972, and is, in that sense, out of date. But, once again, it is a criticism of the subject and not the author that being up to date is considered so important in AI. In a mature science, the basics could be absorbed perfectly well from a four-year-old textbook. In so far as abstract problem-solving techniques, resolution theory in theorem proving, and techniques of the same vintage in vision, are fundamental building blocks of the subject (and to some extent it is undeniable that they are), then they are well and clearly presented here.

The book aims to fill the gap between a wholly popular account of AI research and the style of the technical literature; and is intended to lead anyone interested and armed with 'High School' maths to that literature. It does it rather well, and could be a useful

book for first-year University courses in computer science or psychology.

Its concessions to laymen are among its best bits. Raphael begins by tackling what he calls "myths about computers": the Arithmetic Myth and the Stupid Computer Myth, namely that computers can do only what they are programmed to do, which is true but highly misleading. While he was at it he should probably have tackled the contrary myths: that computers are socially all-powerful and as clever as we are. The latter is just as likely to be believed by the man in the street at the moment as the "myths" Raphael does tackle. The book's subtitle, too, *Mind Inside Matter*, is peculiarly unfortunate for an author concerned to debunk myths.

Another bonus is the well illustrated chapter on the history of robots, which contains the marvellous revelation (p 253) that "several key figures in contemporary computer history"—Von Neumann, Wiener and Minsky—are all descendants of the Maharal: the fifteenth-century rabbi of Prague who made, and gave life to, a 'golem', an artificial man made from the clay of a river bank. □

Yorick Wilks has worked on Artificial Intelligence and natural language understanding at Cambridge, Stanford and in Switzerland. He is at present a Senior Visiting Fellow at the University of Edinburgh, UK.

Matrix isolation

Matrix Isolation: A Technique for the Study of Reactive Inorganic Species. By Stephen Cradock and A. J. Hinchcliffe. Pp. 144. (Cambridge University: Cambridge, London and New York, October 1975.) £6.20.

Now acknowledged as one of the primary methods of investigating molecules which are short-lived in conventional conditions, matrix isolation has not lacked reviewers, but books about it have been few and relatively specialised. The present authors are to be commended therefore on producing a compact and eminently readable account of matrix isolation which can easily be digested by undergraduate and graduate students unfamiliar with the technique. The formation and nature of a matrix, interactions within that matrix, and the technical aspects of matrix-isolation experiments are treated at a simple, descriptive level; specific examples are discussed in the closing chapters. There is a short subject index and a cameo-sized bibliography that is bound to disappoint anyone with more than passing interest in the subject.

Assuming that the reader is familiar with much basic physics, chemistry and spectroscopy, the authors have aimed to show how matrix-isolation experi-

ments may be accomplished. Assuredly, theirs is a reasonable objective, but the result seems to me overburdened with empiricism, and addicted to assertions unsupported either by argument or by apt example. Without a broader appreciation of the purpose and possibilities of the technique, the reader might be excused for wondering whether the technical mountain has not given birth to a chemical mouse. This doubt is not altogether dispelled either by the range or by the treatment of the examples selected. Certain it is that the authors take a healthily critical view of the results of matrix-isolation experiments: whether those results signify much outside their immediate context has to be taken on trust. If the figures would have repaid a greater expenditure of imagination and trouble, the way in which the story is told recalls Mark Twain's character: "there was things which he stretched, but mainly he told the truth". Mainly this is a sound and worthwhile addition to the chemical literature: it might have been more than that.

A. J. Downs

Dr Downs is a Lecturer in the Department of Inorganic Chemistry at the University of Oxford, UK.

Catecholamine transport systems

The Mechanisms of Neuronal and Extraneuronal Transport of Catecholamines. Edited by David M. Paton. Pp. xi+370. (Raven: New York, February, 1976.) \$27.50.

THE discovery by Axelrod and his colleagues that radioactively labelled catecholamines are transported into sympathetically innervated tissues has led to a much better understanding of the physiology and pharmacology of adrenergic neurotransmission, and the early work was summarised in 1967 in what is now becoming a classic book *The Uptake and Storage of Noradrenaline in Sympathetic Nerves* by L. L. Iversen. It is particularly welcome, therefore, that we are brought up to date by the book under review. The editor, in an historical introduction, also reminds the reader that possibly the earliest suggestion that circulating adrenaline might be transported into nerve endings was made by J. H. Burn

in 1932 and first shown experimentally in 1943 by Raab.

The book contains 17 chapters contributed by well-known workers in the field and covers the major catecholamine transport systems known, those across the membranes of sympathetic neurones, of synaptic vesicles and of non-neuronal cells in a variety of tissues. It is of fairly specialised interest, but most workers who study catecholamines and other neurotransmitters will want a copy in their library. Unfortunately, there are some inconsistencies in the book between different authors. Several define precisely what is meant by the term 'uptake' and their definition is welcome; but other authors in the book use the term loosely and are less critical. Finally, the title was not a good choice, for only one chapter covers what is generally understood by the word 'mechanism': the way in which the catecholamine molecule passes across a membrane.

A. D. Smith

Dr Smith is a Lecturer in the Department of Pharmacology at the University of Oxford, UK.

Hydrolysis equilibria of metal cations

The Hydrolysis of Cations. By Charles F. Baes, Jr, and Robert E. Mesmer. Pp. xxi+489. (Wiley-Interscience: New York and London, May 1976.) \$49.40; £27.66.

THE first three chapters of this book will be useful to anyone who wishes to acquire a good background knowledge before beginning a study of the complicated hydrolysis equilibria of metal cations. Methods of measurement (chiefly potentiometric) and the interpretation and treatment of data are covered systematically and in a clear style. The authors conclude this section of the book (pp69-70) with a slightly philosophical attempt to answer the question: How certain can we be that the scheme of equilibria that "best" fits the data is indeed the correct one? The meaning to be attached to the word "correct" in this question would seem to be open to a lengthy discussion among philosophers of science.

The next fourteen chapters are devoted to accounts of specific cations, covering almost all the appropriate elements in the periodic table. This part of the book fulfils the authors' object of assembling in convenient form a great deal of information, most of which was published during the 25 years up to 1974. It is not claimed that the information on any particular topic is complete, but each section (together with the references—there are about 900 altogether) provides a good starting point for further study. In the last chapter a brave attempt is made at the difficult task of surveying and rationalising the variety of behaviour described earlier in detail.

There is no conventional subject index but there is a single-page alphabetical index of hydrolysing species, a chapter index in the form of a periodic table and a ten-page list of contents. The book should therefore be quite convenient to use, but the casual reader should not overlook the summary of symbols and definitions. These are not always conventional, and they include the rather unexpected definition, $pH = -\log[H^+]$. The standard of production is high—as is the price. It is a book for libraries rather than for the individual buyer.

J. S. Coe

Dr Coe is a Senior Lecturer in the Department of Chemistry at King's College, University of London, UK.

Spectroscopy updated

Spectroscopy. Vol. 1: Pp. 304. Vol. 2: Pp. x+362. Vol. 3: Pp. x+324. (Science Paperbacks.) Edited by B. P. Straughan and S. Walker. (Chapman and Hall: London; Halsted: New York, June 1976.) Paper £5.50 each volume; hardback £9 each volume.

THIS set of three volumes consists of a major reform of *Spectroscopy* by S. Walker and H. Straw issued in two volumes in 1961-62. It covers most of non-nuclear spectroscopy with an approach favouring the molecular aspects rather than deep fundamental theory. Each of the nineteen chapters has its individual authorship, although in some places recognisable parts of the earlier work re-appear. There is a modest amount of cross reference, a degree of common style and no serious duplication apart from the deliberate repetition of an appendix containing some more mathematical features in both volumes 2 and 3. The editors have been effective in imposing SI where units are required but they have not had an eagle eye on the associated algebraic forms, many of which lack a factor ($1/4\pi\epsilon_0$) to be consistent with SI. They also eschew $\hbar$ in favour of the cumbersome $h/2\pi$.

Volume 1 contains atomic spectroscopy and various forms of spin resonance including quadrupole nuclei and the Mössbauer effect. In this volume,

nuclear magnetic resonance is only allocated 64 pages which seems light for balance and solids are largely omitted. Volume 2 contains the rotation and vibration features and a chapter on group theory which is at a deeper level than is common in such student texts; the chapter on force constants is more appropriate to this scientific level. The chapter on the far infrared provides interesting experimental detail; instrumentation elsewhere is inevitably briefly covered. Volume 3 covers essentially electronic molecular spectroscopy and includes a special chapter on photoelectron spectroscopy and another on astrophysics and astrochemistry.

This replacement is timely and will be widely welcomed by those who found the earlier edition valuable. The books are somewhat lengthy in parts for chemistry or physics undergraduates, but the volumes should be available in the libraries they use. At MSc level—or possibly undergraduate chemical physics—it would be very suitable; and also as an introduction to research. It can be strongly recommended for these purposes, although it retains a slightly old fashioned approach to quantum theory which restrains the enthusiasm of this reviewer.

D. H. Whiffen

Professor Whiffen is Head of the Department of Physical Chemistry at the University of Newcastle upon Tyne, UK.

Electron donor-acceptor complexes

Catalysis by Electron Donor-Acceptor Complexes: Their General Behaviour and Biological Roles. By Kenzi Tamaru and Masaru Ichikawa. Pp. viii+208. (Kodansha: Tokyo; Wiley: New York and London, March 1976.) \$23.40; £11.70.

IN view of the large number of recent books and reviews dealing with various aspects of electron donor-acceptor (EDA, or charge-transfer) complexes, one must ask whether this book contributes anything new. Unfortunately, aside from one chapter, the answer is no.

The central theme of the book is the chemical properties of EDA complexes, including their reactions, catalysis by EDA complexes, and the biological importance of EDA complexes. Chapters 1 (Introduction, 8 pages) and 2 (Formation of EDA Complexes, 44 pages) present a brief general introduction to the subject of EDA complexes. Most of this material is covered more clearly and accurately in earlier books and reviews.

Chapter 3 (Homogeneous Catalysis by EDA Complexes, 40 pages) discusses reactions of EDA complexes and catalysis by complexing in solution. This chapter contains numerous inaccurate and confusing statements and is neither complete nor up to date. Again, this subject is covered much more adequately in any one of several recent books and reviews.

Chapter 4 (Heterogeneous Catalysis by EDA Complexes, 66 pages) deals with the subjects of adsorption through EDA complexing and by EDA complexes, and catalysis by solid EDA complexes. This topic, being the main interest of the authors, is covered in much more detail with numerous references to recent work. I found the chapter interesting and relatively free from errors. No comparable review of these subjects is available elsewhere.

Chapter 5 (Role of EDA Complexes in Biochemical Reactions, 40 pages) is again neither complete nor up to date and the subject is covered more adequately elsewhere.

A very substantial fraction of Chapter 5 as well as smaller parts of Chapters 2 and 3 are copied almost verbatim from earlier books and reviews. Perhaps this is why, with the exception of Chapter 4, there are few references later than 1968.

In summary, the only very useful part of this book is the chapter dealing with heterogeneous catalysis. Only persons actively interested in this area

are likely to consider paying \$23.40 for a 66-page review on the subject.

Allan K. Colter

Allan K. Colter is Professor of Chemistry at the University of Guelph, Guelph, Ontario, Canada.

Vibrational spectroscopy

Vibrational States. By S. Califano. Pp. xii+335. (Wiley-Interscience: London and New York, April 1976.) £16.75; \$34.50.

THIS is a textbook on the theory of vibrational spectroscopy for graduate students. It evolved from a lecture course given by the author in the University of Florence, and is restricted to the theoretical treatment of vibrations of isolated molecules.

The book consists of nine chapters, plus two appendices containing group-theoretical character and correlation tables. Chapter 1 is a short introduction to infrared and Raman spectroscopy. Chapter 2 presents the classical theory of small molecular vibrations and includes a very clear account of the separation of translational, rotational and vibrational motion (the Eckart or Sayvetz conditions). The quantum theory is given in Chapter 3; it deals with the one-, two- and three-dimensional harmonic oscillator and with the quantum radiation field. Chapter 4 is concerned with the transformation to internal coordinates and the construction of the F and G matrices for the

force constants and the kinetic energy in terms of the internal coordinates. Chapters 5 and 6 are devoted to the principles of group theory and its applications to molecular vibrations, and Chapter 7 to selection rules in infrared and Raman spectra. Various potential functions, including the general valence force field and the Urey-Bradley force field, are discussed in Chapter 8 which includes a brief description of Coriolis coupling, centrifugal distortion, and the *ab initio* computation of force constants. The final chapter is concerned with anharmonicity and Fermi resonance.

The author has given us a clear and detailed account of the main topics in the theory of molecular vibrations. There are omissions, including vibronic coupling, internal rotation, circular dichroism, and Raman spectra resulting from the anti-symmetric part of the polarisability tensor; and a few obscurities and minor errors, such as the comparison at the foot of p20 of quantities having different dimensions. SI units are not used, nor are Mulliken's recommendations (*J. chem. Phys.*, 23, 1997, 1955) for the choice of molecule-fixed axes; the coordinate frames on p19 are left-handed and some of the diagrams (such as that showing a plane of symmetry on p103) are not clear. But overall this is a good book that will be a useful addition to the well-known texts by Herzberg and by Wilson, Decius and Cross.

A. D. Buckingham

A. D. Buckingham has been Professor of Chemistry at the University of Cambridge since 1969.

Environmental dictionary

Dictionary of Environmental Terms. By Alan Gilpin. Pp. 191. (Routledge and Kegan Paul: London and Henley, September 1976.) £3.50.

THIS book is attractively produced, easy to use, and has some very useful features: the list of abbreviations at the beginning (although why does the MW=megawatt come between p.p.m. and its expansion?) and the Appendix carrying the Statement of the Stockholm Conference on the Human Environment (1972). The important criteria in reviewing such a book are: "Does it contain puzzling words that the student/layman is likely to encounter?" and "Are the definitions sound?". The range of words is admirably broad, but there are some omissions, especially of

biological terms. No 'biotic index', 'sere' or 'ecotype', for instance, and only 'meteorological stability'. On the pollution side 'fanning' of plumes appears, but no 'slug' or 'slick'; and although 'effluent charge' is included 'consent' or 'consent conditions' is not.

'Qualitative and quantitative analysis' probably need not have been included, and the long description of Boulding's green stamp plan for creating a market in procreation, although intriguing, is of only marginal importance.

Some of the explanations are too discursive, more appropriate to a gazetteer than a dictionary. In spite of these criticisms, however, this is a helpful book reasonably priced.

Pauline K. Marstrand

Pauline Marstrand is a Senior Fellow in the Science Policy Research Unit, University of Sussex, UK.

obituary

With the death of **Professor J. C. Slater** at the age of 75, America, and indeed the world, has lost one of its most influential and active practitioners of theoretical physics. For over fifty years Slater produced a continuous and substantial flow of research work, most of it concerned with those aspects of matter which depend primarily on the electronic properties of atoms; treating first the properties of individual atoms as revealed by the multiplet structure of their spectra, and later the properties of atoms in molecular combinations and in the solid state.

With the advent of wave mechanics he quickly realised that all such properties are determined by the many-electron wave function of the system, and therefore directed his attention to the invention of suitable approximations and mathematical methods for its calculation. In his search for the wave functions, their eigenvalues, and the density of states they imply, he led a numerous band of gifted students many of whom (now somewhat advanced in years) have followed the trail which he blazed and, with more powerful computers and more refined techniques, have achieved even better results. Surely no greater tribute can be paid to a scientist than the successful continuation of his work by succeeding generations.

John Clarke Slater was born on December 22, 1900 into an academic family; his father was professor of English at the University of Rochester. John, after taking his bachelor's degree at this university, went on to Harvard as a graduate student to work under the direction of P. W. Bridgman. In 1922 he travelled to Europe and during a stay of a few months in Copenhagen he became part author, with Bohr and Kramers, of a well-known paper on the quantum theory of radiation. Although

the conclusions of this paper proved to be incorrect, it was influential for a time, and for a young man of 23 the association with such famous names was a happy augury indeed.

In 1929 Slater published one of his most important papers which dealt with the theory of multiplets in complex spectra. Following the work of F. Hund and applying the newly developed wave mechanics he was able to simplify and extend the calculations of the multiplet structure of atoms with many electrons. The basic concept in this work was the expression of the total wave function as a determinant of the single electron wave functions including their spin states. Such determinants have ever since been known as Slater determinants and have found applications in many fields, for example, chemistry and metal physics.

Following the publication of Heisenberg's theory of ferromagnetism and Bloch's famous paper on the quantum mechanics of electrons in crystal lattices, Slater wrote a paper in 1930 entitled *Cohesion in Monovalent Metals*. The significance of this paper in Slater's scientific life is that it marks his introduction into what is now known as solid state physics and which was to remain his principal interest for over forty years. As with many other physicists, however, the years of the second world war caused an interruption in Slater's academic research. During these years he was active in organising the Radiation Laboratory at M.I.T. and was involved with research concerning the principles of magnetron design. For this work he was awarded a Presidential Certificate of Merit.

After the war Slater returned to his interests in solid state physics and began a series of calculations aimed at improving the accuracy of wave functions and the energy levels of electrons

in metals. He developed the method introduced by Wigner and Seitz so that it could be applied to states of higher energy and used the resulting eigenvalue spectrum to construct curves of the density of states. Later he introduced the augmented plane wave method which was widely used by his own students and other physicists. This work, carried out mainly in the decade 1950-60, is not to be judged by the accuracy, or inaccuracy, of particular results but by the thrust which it gave to the advance in this sector of the subject.

Slater was a most prolific writer. Besides about one hundred original papers he wrote eleven books, a few in collaboration with N. H. Frank, but mostly alone, and in addition produced many survey articles, including one giving a detailed history of the development of solid-state and molecular theory. A notable feature of Slater's pedagogic writings is the extraordinary complete bibliography which is invariably attached to each book or article. Evidently very little escaped his attention. It seems clear that he wrote with great facility and speed and that he maintained a confidence in his knowledge and understanding of physics throughout his life.

Other aspects of Slater's life and character, for example, his administrative ability which enabled him to take over the headship of the physics department of M.I.T. at the early age of 29, and his gifts as a teacher, can be dealt with adequately only by a close colleague, and in an introductory passage to the volume of articles published to commemorate his 65th birthday, Professor Philip Morse pays a warm tribute to Slater with regard to exactly these qualities. Clearly, Slater was a man of quite exceptional stature in many respects.

H. Jones

announcements

Meetings

March 28-30, **Nuclear Magnetic Resonance in Biology**, Oxford (Dr R. A. Dwek, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK).

April 4-6, **Microcalorimetry in Biology**, London (3rd International Symposium

on Microcalorimetry, LKB Instruments Ltd., LKB House, 232 Addington Road, Selsdon, South Croydon, Surrey CR2 8YD, UK).

April 25-27, **Bioengineering**, Fort Collins (Deadline for abstracts: January 1) (Dr C. W. Miller, Department of Physiology and Biophysics, Collaborative Radiological Health Laboratory, Colorado State University,

Fort Collins, Colorado 80521).

April 27-29, **Food, Fertilizer and Agricultural Residues**, Syracuse, New York (Waste Management Conference, Cornell University, 207 Riley-Robb Hall, Ithaca, New York 14853).

May 21-25, **Carboniferous Stratigraphy and Geology**, Urbana-Champaign (Dr Mackenzie Gordon, U.S. Geological Survey, Washington D.C.).